Functional Interactions of 14-3-3 Proteins with Phospholipase D and the M_3 Muscarinic Receptor

by

Daniel M. Collins

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This thesis is dedicated to my parents, Gillian and Michael, to my grandmother Doreen and to the memories of Leslie, Margaret and especially Eddie.

Declaration of Originality

I (Daniel Collins) declare that, unless otherwise stated, this thesis represents my own work and was composed by me. No part of this work has been, or is being submitted for any other degree or qualification.

Daniel Collins

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Abbreviations

A_λ	absorbance
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride.
Ach	acetylcholine
ATP	adenosine triphosphate
Bq	Becquerels
BSA	bovine serum albumin
C-	carboxy-(terminus)
cAMP	cyclic 3,5-adenosine monophosphate
CCh	carbamyl chloride (carbachol)
CO_2	carbon dioxide gas
COS 7	green monkey kidney fibroblast cell line
cDNA	complementary deoxyribonucleic acid
°C	degrees Celsius
CHAPS	3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate
DAG	sn 1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
EBSS	Earle's balanced salt solution
eCFP	enhanced cyan fluorescent protein
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FLIM	fluorescence lifetime imaging
FRET	fluorescence resonant energy transfer
GST	glutathione s-transferase
h	hours
HA	haemagluttinin
HBSS	Hank's buffered saline solution
HCl	hydrochloric acid
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kiloDaltons
λ	wavelength
l	litre
LB	Luria Bertani
M_3	muscarinic receptor type 3
M	molar
MES	2-(N-morpholino) ethane sulfonic acid
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
mm	millimetre
MOPS	3-(N-morpholino) propane sulfonic acid
μg	microgram
μl	microlitre

μm	micron
μM	micromolar
N-	amino-(terminus)
NaCl	sodium chloride
NCS	normal calf serum
ng	nanogram
nm	nanometre
nM	nanomolar
N-MeQNB	N-methyl-quinuclindinyl benzilate
O_2	oxygen gas
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline solution
PDBu	phorbol 12,13-dibutyrate
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D (protein kinase Cμ)
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
pmoles	picomoles
PtdBu	phosphatidylbutanol
InsP	inositol phosphate
PVDF	polyvinyldifluoride
RNA	ribonucleic acid
RNase	ribonuclease
s	second(s)
S.D.	standard deviation
SDS	sodium dodecyl sulphate
S.E.M.	standard error of mean
Tris	Tri (hydroxymethyl)-aminomethane
UHP	ultra high purity (resistance > 17 mega-ohms)
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Abstract

14-3-3 proteins are a family of small, acidic, scaffolding and adapter proteins, which have been implicated in cell cycle regulation, apoptosis and signal transduction mechanisms. There are seven isoforms of 14-3-3 (β, η, γ, ε, τ/θ, σ and ζ) that form hetero- and homodimers *in vivo*. Recently, 14-3-3 has been shown to associate with members of the heptahelical, plasma membrane spanning G-protein coupled receptor (GPCR) superfamily. GPCRs mediate neurotransmitter and other extracellular agonist-evoked activation of intracellular effectors and signalling cascades. Some of these effector mechanisms lead to the activation of phospholipase D (PLD). Mammalian PLD isoforms catalyse the hydrolysis of phosphatidylcholine, forming choline and phosphatidic acid, a novel second messenger molecule. 14-3-3 dimers associate with other proteins containing specific target motifs, including an RSxpSxP motif (where pS is phosphoserine), or an unphosphorylated WLDLE/DALDL motif. We recognised that the former motif is present in mammalian PLD1 at residues 712-717 and therefore have investigated whether 14-3-3 isoforms associate with PLD and GPCRs to provide a functional role in intracellular signalling. It was shown, using *in vitro* GST-fusion protein pull-downs and co-immunoprecipitation that in COS 7 cells, 14-3-3 associates with the M_3 muscarinic receptor. 14-3-3 was also demonstrated to associate with PLD1, and to a lesser extent PLD2, in an isoform-dependent manner. The effect of PLD activation by protein kinase C (PKC) on this interaction was investigated using the aforementioned techniques and confocal microscopy. Furthermore, in whole cell signalling assays, the overexpression of different 14-3-3 isoforms selectively modified PKC or GPCR-mediated activation of PLD. In addition, PLD was found to physically associate with the M_3 receptor. The implications of these interactions for physiological signalling by the M_3 receptor and PLD are discussed.

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Introduction

Introduction

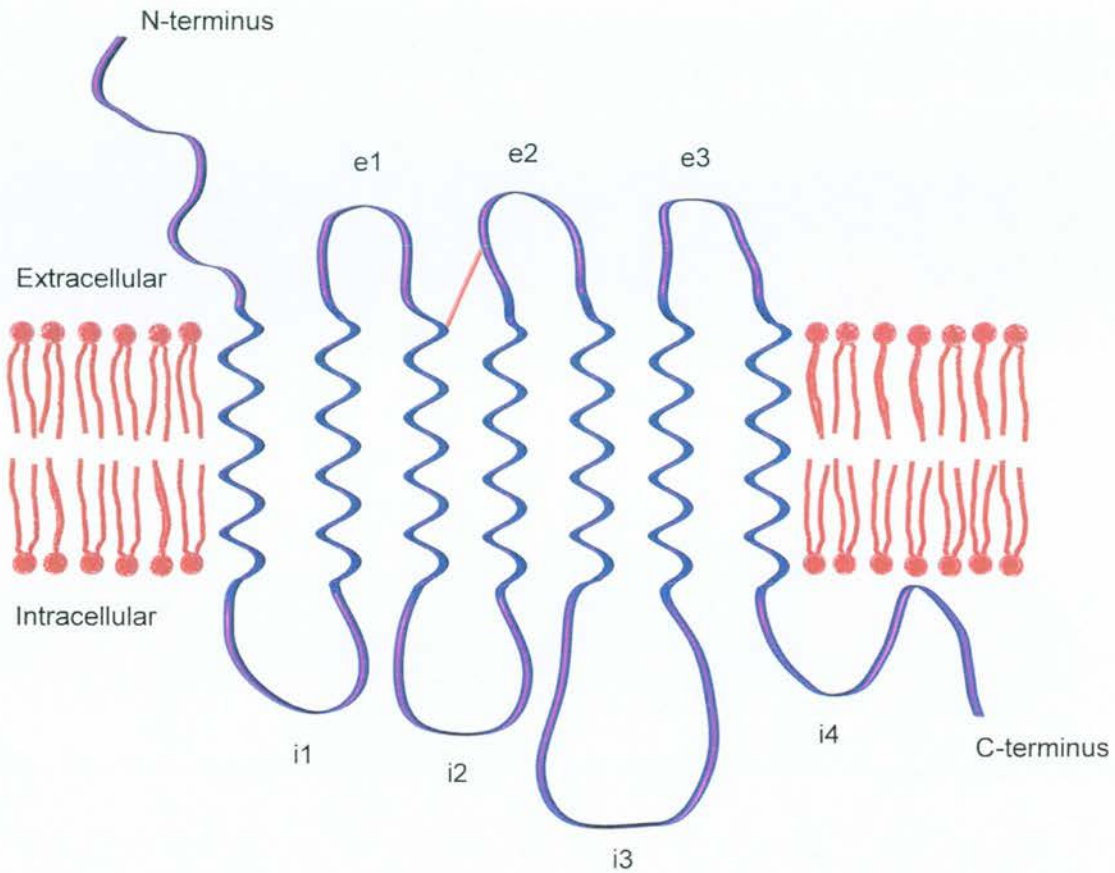
Regulation of many biological processes depends on cellular recognition of the external environment and the generation of an appropriate response. Cell surface receptors play an important role in transducing this external information into intracellular signals. The cell surface receptors can be divided into superfamilies, depending on the mechanism of signal transduction. Amongst these are ionotropic ligand-gated ion channels, G protein-coupled receptors and receptor tyrosine kinases. Over 50% of current therapeutic drugs act on members of the G protein-coupled receptor superfamily (Archer *et al.*, 2003; Karnik *et al.*, 2003) and they remain a very important pharmacological target.

The G protein-coupled receptor

The G protein-coupled receptor (GPCR) superfamily is the largest family of the cell surface receptors and amongst the largest families of proteins in the human genome, comprising of more than 700 gene products (Lander *et al.*, 2001; Venter *et al.*, 2001). The G protein-coupled receptor itself remains one of the most important pharmacological targets for drug design, however the continual process of elucidating novel effectors has yielded ever more complexity for understanding the full range of specific signalling events that can be regulated by these receptors. All GPCRs are integral membrane proteins that have a similar architecture consisting of an extracellular amino terminal domain, a seven α-helix plasma membrane-spanning domain, three extracellular and three intracellular loops and terminating at an intracellular carboxy-tail domain (Figure 1.1) (Dohlman *et al.*, 1987; Baldwin, 1993). The template for determining the structural aspects of G protein-coupled receptor topology was originally based on nuclear magnetic resonance (NMR) data, electron cryo-microscopy and crystal structure of the transmembrane spanning archaebacterial proton pump, bacteriorhodopsin, from *Halobacterium halobium* (Henderson and Unwin, 1975; Hayward and Stroud, 1981; Spohn and Kimmich, 1983; Frankel and Forsyth, 1985; Lewis *et al.*, 1985; Pebay-Peyroula *et al.*, 1997). Areas of sequence similarity between

bacteriorhodopsin and other GPCRs were found to exist within the seven transmembrane spanning α-helices of bacteriorhodopsin and suggested that GPCRs probably had a similar structural topology, with the presence of a seven transmembrane spanning domain (Findlay and Eliopoulos, 1990; Henderson and Schertler, 1990; Pardo *et al.*, 1992). Refinements in the structural analysis of GPCR topology were made using the abundant photoreceptor of the rod cells in the eye, rhodopsin (Daemen, 1978; Findlay *et al.*, 1981; Argos *et al.*, 1982; Findlay *et al.*, 1984; Dratz *et al.*, 1985; Longstaff *et al.*, 1986). Rhodopsin, used as a prototypical GPCR, coupled with further developments allowed by the increasing power of computational modelling and continuing biochemical and molecular biology studies, yielded a high level of information regarding the structural characteristics and possible structure-function relationships that exist with signalling mechanisms of other GPCRs (Dixon *et al.*, 1986; Kubo *et al.*, 1986; Peralta *et al.*, 1987b; Henderson and Schertler, 1990; Hollenberg, 1991; Hargrave and McDowell, 1992; Baldwin, 1993; Donnelly *et al.*, 1994; Unger *et al.*, 1997). The inactivated (11-*cis*-retinal) rhodopsin photoreceptor was solved by X-ray crystallography in 2000 (Palczewski *et al.*, 2000) and the structure appeared to verify alternative experimental and modelling methods that had been used to determine the nature of GPCR structure-function relationships (Baldwin, 1993; Farahbakhsh *et al.*, 1995; Unger *et al.*, 1997; Karnik *et al.*, 2003). The seven transmembrane spanning α-helices are organised in an anticlockwise bundle (visualised from the extracellular side), the third transmembrane spanning helix is almost central to the bundle and changes orientation from the extracellular to the intracellular side of the membrane. Furthermore, an amphipathic α-helix, parallel to the plane of the membrane was identified distal to the seventh transmembrane spanning α-helix (and correlates to the i4 region), which may be important for G protein coupling (Figure 1.2) (Bourne, 1997; Wess, 1998; Palczewski *et al.*, 2000).

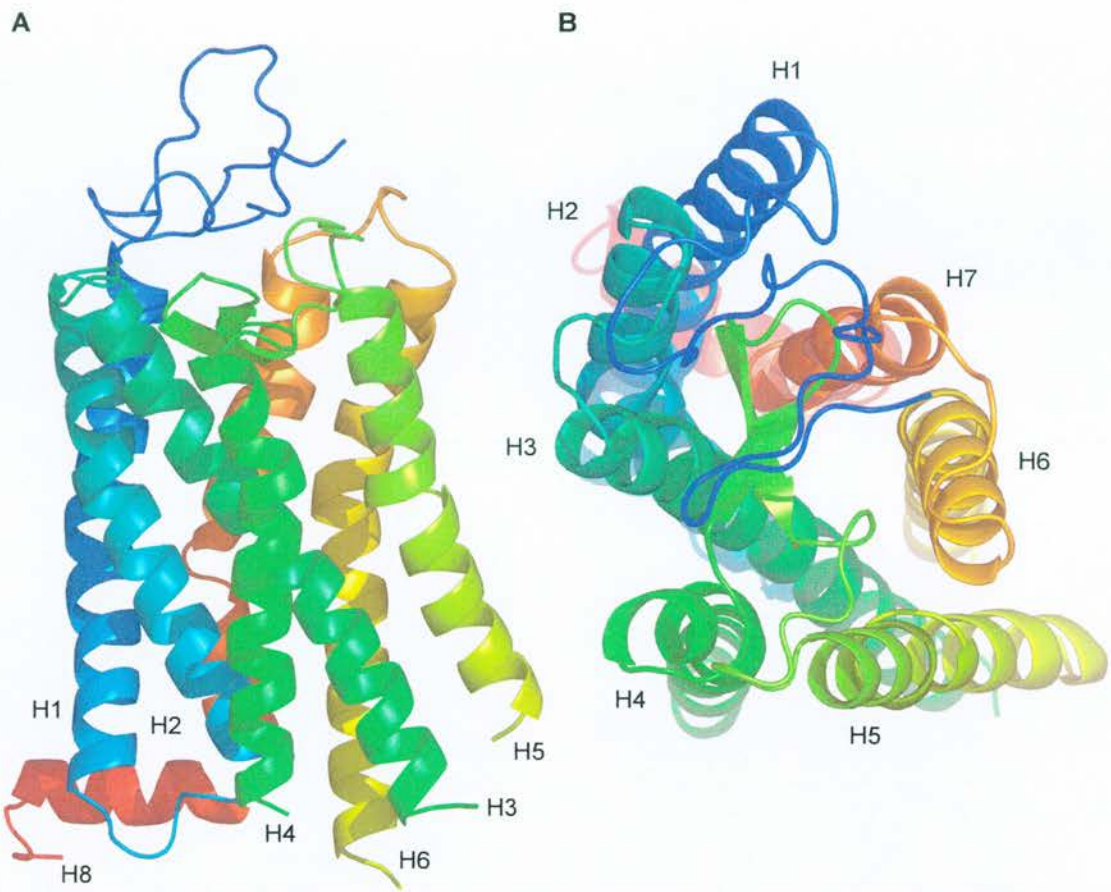
Figure 1.1



The topology of a typical G protein-coupled receptor.

A two dimensional diagram of the topology of a generic G protein-coupled receptor (GPCR) (purple) bounded by lipid groups of the plasma membrane (red). The extracellular amino-terminal domain, seven transmembrane spanning domain and intracellular carboxy-terminal tail domain are shown, along with the three intracellular (i1-3) and extracellular (e1-3) loops (with disulphide bridge between the third membrane spanning helix and the second extracellular loop). The palmitoylation of the tail (present on most GPCRs) causes a pseudo fourth intracellular loop (sometimes referred to as i4), which is made up of a short α-helix, orientated parallel to the plasma membrane in rhodopsin. In the folded protein, the transmembrane regions form a barrel into which the agonist can bind (see Figure 1.2).

Figure 1.2



The crystal structure of rhodopsin, a template for the G-protein coupled receptor.

The solved crystal structure for the seven transmembrane spanning helices of the rhodopsin hepatahelical receptor at 2.6 Å resolution shown in ribbon form, from the side (A) and from the extracellular (B) views (Berman *et al.*, 2000; Okada *et al.*, 2002). The peptide chain is rainbow colour coded, from the blue N-terminus to the red C-terminus. The seven transmembrane anticlockwise α-helix bundle is apparent (with helices labelled H1-7), as is the extracellular amino- and intracellular carboxy-terminal domains (with the additional amphipathic α-helix (H8) parallel to the plane of the membrane). The change of orientation of the third transmembrane helix (from the extracellular (top) to the intracellular (bottom) surface) is also apparent.

Classification of the GPCR superfamily has been attempted using a number of different phylogenetic and structural categorisations (Donnelly *et al.*, 1994; Kolakowski, 1994; Fredriksson *et al.*, 2003). This task has been made more efficient with the sequencing of the human genome, as all the gene products encoding GPCRs have been determined (Lander *et al.*, 2001; Venter *et al.*, 2001). The original nomenclature of receptor classes (A-F) includes those receptors from many different types of organisms (Kolakowski, 1994). An alternative, recent classification is based on the GRAFS family system (Fredriksson *et al.*, 2003; Karnik *et al.*, 2003). This system includes only those GPCRs from the human genome and, like the class categorisations, compares homology of the transmembrane spanning domains of the GPCRs to determine a dendrogram of the most related receptors. This yields five main families of GPCRs within the human genome and these are discussed below. Although all of the families have a similar structural topology and nearly all of the receptors within each class have been shown to couple to heterotrimeric G proteins, they each share relatively little sequence similarity to each of the other families of GPCRs (Foord, 2002). For convenience, both the original mammalian classes and the human GRAFS family nomenclature are provided.

The rhodopsin-like receptors

The rhodopsin-like (family R, or class A) receptors are closely related in homology to the photon receptor, rhodopsin (Dixon *et al.*, 1986), they are the largest class of GPCRs (Fredriksson *et al.*, 2003) and have been the most widely studied. The rhodopsin-like receptors number around 700 (241 non-olfactory) in humans, have been further subdivided into four major groups and comprise of many subfamilies including the olfactory (group δ), biogenic amine, prostanoid and cannabinoid receptors (group α), endothelin and neuropeptide Y (group β) and opioid and somatostatin receptors (group γ) (Dixon *et al.*, 1986; Kubo *et al.*, 1986; Strange, 1993; Drutel *et al.*, 1995; Narumiya and FitzGerald, 2001; Foord, 2002; Ronnett and Moon, 2002; Fredriksson *et al.*, 2003). The bovine rhodopsin

receptor was fully sequenced and cloned in 1983 (Nathans and Hogness, 1983), following previous sequence analysis of parts of the protein (Hargrave, 1977; Hargrave and Fong, 1977; Pellicone *et al.*, 1981). The sequence of the human form of the rhodopsin receptor followed in the following year (Nathans and Hogness, 1984). The ligand-activated β-adrenergic receptor was cloned in 1986 by Dixon and colleagues and it appeared to bear structural homology with the rhodopsin photoreceptor (and implied that a number of other cell surface receptors may also have similar features) (Dixon *et al.*, 1986). Since the cloning of the rhodopsin and β-adrenergic receptor, many GPCRs and their splice variants have been characterised from their gene products. The most extensively studied of the rhodopsin-like GPCRs are the subfamilies of the biogenic amine receptors – the adrenergic receptor (α and β adrenenoceptors) (Lefkowitz *et al.*, 1976; Lefkowitz *et al.*, 1981; Dixon *et al.*, 1986), the muscarinic acetylcholine receptors (M receptors) (Kubo *et al.*, 1986; Bonner *et al.*, 1987), the dopamine receptors (D receptors) (Strange, 1993) and the 5-hydroxytryptamine (5-HT) receptors (Hoyer *et al.*, 1994; Hoyer *et al.*, 2002).

All rhodopsin-like receptors contain highly conserved regions within their structure. These include a disulphide bridge, found in nearly all GPCRs, formed by cysteine residues that link the second extracellular loop to the third transmembrane spanning helix (Pedersen and Ross, 1985; Dixon *et al.*, 1987; Karnik and Khorana, 1990; Schertler *et al.*, 1993). There is a conserved Asp-Arg-Tyr (DRY) motif at the intracellular junction of the third transmembrane helix, which has been proposed to be involved in the efficient coupling of the GPCR to the heterotrimeric G proteins. Mutations in these residues have been shown to constitutively activate the signalling by the α_1- and β_2-adrenergic receptor (Scheer *et al.*, 1996; Rasmussen *et al.*, 1999), the A3 adenosine receptor (Chen *et al.*, 2001) and the GnRH receptor (Arora *et al.*, 1997). However, a conflicting report has indicated that mutation of the arginine in the DRY motif of the β_2-adrenergic receptor does not affect activity (Seibold *et al.*, 1998). In addition a conserved NPxxY motif at the distal end of the seventh

transmembrane spanning helix has been shown to be involved with the GPCR activation of the small G proteins ARF and Rho (Mitchell *et al.*, 1998) and may be necessary for the activation of the Gq heterotrimeric G proteins in the stimulation of PLC activity (Gales *et al.*, 2000). The NPxxY domain has also been demonstrated to be important for efficient internalisation of some GPCRs such as the δ-opioid receptor (Kramer *et al.*, 2000), the type II vasopressin receptor (Bouley *et al.*, 2003), the bradykinin receptor (Kalatskaya *et al.*, 2004) and the formyl-peptide receptor (Gripentrog *et al.*, 2000; He *et al.*, 2001), but not for others such as the angiotensin II type 1 receptor (Thomas *et al.*, 1995; Hunyady *et al.*, 2000). Furthermore, the side chains of the amino acids on the internal face of the transmembrane spanning domain have been shown to be important for providing selectivity of ligand binding. Amongst these is an aspartate residue in the third transmembrane spanning helix (at position 113 in the β_2-adrenergic receptor and position 148 in the M_3 receptor) that is conserved within the biogenic amine liganded GPCRs and which is thought to coordinate an important ionic interaction with the nitrogen of the ligands (Dixon *et al.*, 1987; Strader *et al.*, 1987; Strader *et al.*, 1988; Matsui *et al.*, 1989; Strader *et al.*, 1989b). Moreover, amine GPCRs also have conserved serine or threonine residues in the helix barrel, which potentially coordinate the hydroxyl groups of the aromatic ring of the ligand (Strader *et al.*, 1989a; Wess *et al.*, 1991). There is a conserved proline residue, present in the fourth transmembrane spanning helix, which has also been demonstrated to be important for high affinity ligand binding of the M_3 receptor (Wess *et al.*, 1993). Antagonists are thought to coordinate to some of the residues involved in agonist binding, but not to some critical residues involved in receptor activation (Wess *et al.*, 1991). The peptide receptors have a larger extracellular amino-terminal domain, which is thought to coordinate additional interactions with the larger peptide agonists (especially on the third extracellular loop), however the residues within the transmembrane domain remain very important for ligand binding (Bhogal *et al.*, 1994; Schwartz, 1994).

It has been demonstrated that the second and third intracellular loop and carboxy-terminal tail domains in many GPCRs are necessary for coupling to the specific heterotrimeric G proteins (Strader *et al.*, 1989b; Wess *et al.*, 1990; Wu *et al.*, 2000) and small G proteins (Robertson *et al.*, 2001). Regions of the third intracellular loop and carboxy-terminal tail domains have also been shown to be phosphorylated (and desensitised) by G protein receptor kinases and bind the internalising arrestin proteins (see below) (Lohse *et al.*, 1990; Freedman and Lefkowitz, 1996; Krupnick *et al.*, 1997b; Wu *et al.*, 1997; Pitcher *et al.*, 1998). In addition, there is a cysteine residue in the carboxy-terminal domain of many GPCRs that is palmitoylated and which may thereby cause the formation of a pseudo fourth intracellular loop (involving the amphipathic α-helix that was determined by X-ray crystallography) (Wess, 1998; Palczewski *et al.*, 2000).

The secretin-like receptors

The secretin-like (family S, or class B) G protein-coupled receptors have 15 members that all bind large peptides and include the families of the secretin, corticotrophin releasing factor (CRF), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) receptors (Christophe *et al.*, 1988; Lutz *et al.*, 1993; Morrow *et al.*, 1993; Spengler *et al.*, 1993; Foord *et al.*, 2002). The receptors are characterised by a large extracellular amino-terminal domain, which contains a number of conserved residues including a series of cysteine residues thought to form important structurally stabilising disulphide bonds with the extracellular loop domains (Gaudin *et al.*, 1995). Site-directed mutagenesis and chimaeric receptor studies have demonstrated that regions involving both the transmembrane spanning domain (similarly to the rhodopsin-like GPCRs) and the extracellular loop domains contribute to coordinating high affinity ligand binding to the larger peptide agonists (Di Paolo *et al.*, 1999; Unson *et al.*, 2002). Most members of the secretin family have a basic residue followed by a conserved aspartate at the junction between the second transmembrane spanning helix and the proximal region of the first

extracellular loop region and these residues have been shown to be important for ligand binding to the VPAC receptors (Solano *et al.*, 2001; Vertongen *et al.*, 2001; Langer *et al.*, 2003). In addition, some of the conserved cysteine residues of the extracellular domains have also been shown to be important for ligand binding (Gaudin *et al.*, 1995; Harmar, 2001; Unson *et al.*, 2002). A model of the peptide agonist binding mechanism has been proposed in the case of the glucagon and glucagon-like peptide 1 receptor (GLP-1) that identifies the GPCR transmembrane spanning regions as important for coordinating the amino-terminal domain of the peptide agonist, with the amino-terminal domain and extracellular loops of the receptor being responsible for coordinating the central and carboxy-terminal regions of the peptide (Gourlet *et al.*, 1996; Al-Sabah and Donnelly, 2003; Runge *et al.*, 2003). This has been further substantiated by NMR spectrometry data from the N-terminal domain of the corticotrophin-releasing factor (CRF) receptor (Grace *et al.*, 2004).

The regions of the receptor involved in coupling to the heterotrimeric G proteins have been demonstrated to involve the intracellular loop and carboxy-terminal tail domains in a similar way to the rhodopsin-like GPCRs, however the second intracellular loop may be more important then the third (Chicchi *et al.*, 1997; Cypess *et al.*, 1999). There is also proposed to be an equivalent motif to the DRY region, the conserved YL residues, mutations of which were shown to alter G protein activation by the VPAC1 receptor (Tams *et al.*, 2001). One interesting aspect of the secretin-like GPCRs' signalling mechanisms is that all the receptors appear to preferentially couple to the Gs heterotrimeric G protein family to activate adenylate cyclase and increase intracellular cAMP, however many can also couple to other family heterotrimeric G proteins (to activate phospholipase C, for example) (Harmar, 2001). Furthermore, the receptors recruit G protein-receptor kinases and arrestin proteins in a similar way to rhodopsin-like GPCRs to desensitise the agonist-stimulated signalling (Shetzline *et al.*, 1998).

The metabotropic glutamate-like receptors

The glutamate-like (family G, or class C) receptors have 15 members and include the metabotropic glutamate receptor (mGluR) family, the metabotropic $GABA_B$ receptors, Ca^{2+} sensing receptors, pheromone and group-1 taste receptors (Houamed *et al.*, 1991; Masu *et al.*, 1991; Conn and Pin, 1997; Kaupmann *et al.*, 1997; Couve *et al.*, 2000; Pin *et al.*, 2003). These GPCRs are characterised by a very large extracellular amino-terminal (Desai *et al.*, 1995). The amino-terminal contains a conserved Venus Flytrap Module (VFTM) used for ligand recognition (O'Hara *et al.*, 1993; Takahashi *et al.*, 1993; Tones *et al.*, 1995) and (in all except the $GABA_B$ receptor) a conserved cysteine rich domain that connects the VFTM to the transmembrane spanning domain (Pin *et al.*, 2003). The VFTM domain shares sequence similarity with a family of small molecule-binding bacterial periplasmic transport proteins (O'Hara *et al.*, 1993), and it has been proposed that the family G GPCRs may have evolved from the fusion of an ancestral seven transmembrane receptor with one of these periplasmic proteins (Felder *et al.*, 1999; Pin *et al.*, 2003). The Venus Flytrap Module is so called because it is composed of two regions (or lobes) each composed of a β-sheet surrounded by two α-helices, separated by three linkers, which form a cleft into which the ligand binds (Sack *et al.*, 1989; Galvez *et al.*, 1999; Kunishima *et al.*, 2000). These lobes can be found in an open or closed conformation with an equilibrium constant that favours the open state when the receptor is unliganded and therefore the closed state is stabilised when ligand is bound (Parmentier *et al.*, 2002). It is thought that part of the mechanism of competitive antagonist ligands may be due to the interference with efficient closure of the VFTM lobes (Costantino and Pellicciari, 1996).

The family G receptors were the first GPCRs to be shown to form functional dimers *in vivo* (which has been reproduced subsequently for a number of other family GPCRs) (Romano *et al.*, 1996; Robbins *et al.*, 1999; Milligan *et al.*, 2003). Whilst mGluR and Ca^{2+} sensing receptors form homodimers, $GABA_B$ and taste receptors form heterodimers for full agonist activation (Romano *et al.*, 1996; Kuner *et al.*, 1999; Kunishima *et al.*, 2000; Pin *et*

al., 2003). Dimerisation of the receptors has been shown to involve the VFTM domains (formed by a disulphide bridge and a hydrophobic interaction), a putative transmembrane interaction and the carboxy-terminal tail domains (Bai *et al.*, 1998; Ray *et al.*, 1999; Robbins *et al.*, 1999; Ray and Hauschild, 2000; Tsuji *et al.*, 2000). It has been determined that the receptors do not require two agonist molecules to bind to elicit activation (Galvez *et al.*, 2000), however X-ray crystallography data of the N-terminal domain suggests that the active state of the receptor may be more stable when the VFTM lobes of both receptors in the dimer are closed (Kunishima *et al.*, 2000; Pin *et al.*, 2003). Furthermore, the GPCR dimer does not appear to activate more than one heterotrimeric G protein at any time (however, the functional dimer is still required for G protein coupling) (Galvez *et al.*, 2001; Pin *et al.*, 2003).

The frizzled, adhesion and other G protein-coupled receptors

The family F receptors have 24 members and are comprised of the frizzled/smoothened receptors and the group-2 taste receptors. These receptors have short conserved sequences in transmembrane helices 2, 5 and 7 (Fredriksson *et al.*, 2003). The frizzled receptors control cell proliferation and polarity during metazoan development by binding secreted glycoproteins known as Wnts (Fredriksson *et al.*, 2003). Although they may all be GPCRs, only a few of the members have been shown to couple to heterotrimeric G proteins and they may be constitutively active (Foord *et al.*, 2002). These receptors, along with the family A receptors, were previously classified as belonging to the secretin-like receptors (Harmar, 2001; Foord *et al.*, 2002), however the GRAFS system determines that they have their own functional classification (Fredriksson *et al.*, 2003).

The family A receptors (24 members) are made up of the adhesion receptors (or latrophilins), which are receptors that have the typical transmembrane spanning domain coupled to an amino-terminal domain that contains a functional adhesion-like motif, such as the EGF repeat motif or a mucin-like motif (Fredriksson *et al.*, 2003). The amino-terminal

domains are vastly different in size, ranging from 200 residues up to 2800 residues in the case of the very large G protein coupled receptors (VLGRs) (Foord *et al.*, 2002). Functional activities associated with these receptors include the definition of cell polarity, inhibition of angiogenesis and regulation of the immune system. In addition, some members are allosteric receptors for α-latrotoxin (venom from the black widow spider *Latrodectus mactans*), a powerful secretagogue for insulin (Holz *et al.*, 2000).

Heterotrimeric G protein signalling cascades

The signalling by GPCRs has traditionally focused on the heterotrimeric G protein signalling pathway as the main mechanism for intracellular second messenger activation. The mechanism of a guanine nucleotide dependent protein acting as an intermediary transducer between the GPCR and the activation of intracellular effectors was originally proposed by Rodbell and colleagues (Rodbell *et al.*, 1971). The involvement of a signal transducing GTPase was verified by Limbird and colleagues when they demonstrated that cholera toxin could specifically ADP-ribosylate (and thereby block) a GTPase activity required for the β-adrenergic receptor-dependent activation of adenylate cyclase (Limbird *et al.*, 1979; Limbird *et al.*, 1980; Stadel *et al.*, 1981). It is now accepted that nearly all GPCRs (except for some orphan or family F receptors, see above) couple directly through a heterotrimeric G protein to activate or modulate intracellular effectors (Stadel *et al.*, 1981; Blackmore *et al.*, 1985; Bourne and Sullivan, 1986; Sullivan *et al.*, 1986; Bourne *et al.*, 1987).

The G proteins are made up of a trimer of Gα (~41 kDa), Gβ (~35 kDa) and Gγ (~10 kDa) subunits (Baehr *et al.*, 1982; Northup *et al.*, 1983; Gilman, 1984). The βγ subunits are tightly bound, extremely stable and are consequently regarded as a single functional unit. Currently there are thought to be around twenty gene products encoding Gα subunits, five encoding Gβ and thirteen encoding Gγ (Venter *et al.*, 2001). The number of

various possible subunit combinations is potentially very large and this combinatorial characteristic may yield part of the regulation that is necessary for the specificity of second messenger coupling and activation. The Gγ subunit displays the lowest homology between the isoforms and is acylated at the carboxy-terminal to provide interaction with the lipid membrane. The Gβ subunit structure is dominated by the presence of a β-propeller domain and associates with the Gγ subunit via a coiled-coil interaction (Morris and Malbon, 1999). The Gα subunits are composed of two main domains - a Ras-like GTPase domain that is important for GTPase activity, connected to a helical domain that may be important for effector coupling (Liu and Northup, 1998). The GDP/GTP is bound at the interface of the two domains and a change in conformation at three additional switch regions, which are contacted by the Gβγ subunit, allows GTP/GDP exchange (Morris and Malbon, 1999).

The heterotrimeric G protein family nomenclature is based upon the types of second messenger pathways that the Gα subunits of the G proteins couple to and they can be classified (at least in part) into four main families; Gs, Gi, Gq and G12 (see below). It was traditionally thought that the α-subunits of the G protein could exclusively regulate their downstream effectors and the original family nomenclature arose with this caveat. However, in 1987, Logothetis and colleagues demonstrated that exogenous application of the Gβγ subunit to excised patch clamped atrial membranes caused activation of the muscarinic K^+ current, which is present in the heart (Logothetis *et al.*, 1987). Subsequently Gβγ subunits have been shown to couple to other intracellular effectors such as PLCβ2-3 (Smrcka and Sternweis, 1993; Haga *et al.*, 1994; Luttrell *et al.*, 1995; Pumiglia *et al.*, 1995) as well as to many ion channels (Penington *et al.*, 1993; Krapivinsky *et al.*, 1995; Herlitze *et al.*, 1996; Ikeda, 1996), demonstrating the importance of both the Gα and Gβγ subunits for signal transduction. Furthermore, specificity of Gβγ coupling to the G protein-coupled inwardly-rectifying K^+ channels has been shown to be dependent on the Gβ subunit type, with β1-4

being able to activate the K channel and β5 (and Gα subunits) coupling to inhibit the channel (Lei *et al.*, 2003).

The Gs heterotrimeric G protein family includes the Gαs and Gαolf G proteins. The Gs G protein was the first one characterised by Limbird and colleagues and has been demonstrated to stimulate adenylate cyclase, which catalyses the formation of the second messenger, cAMP, from ATP (Lefkowitz *et al.*, 1976; Limbird *et al.*, 1980; Sullivan *et al.*, 1986). An increase in cAMP levels subsequently activates protein kinase A to mediate other intracellular effects (Miyamoto *et al.*, 1968; Robison *et al.*, 1968; Walsh *et al.*, 1968). Furthermore, the Gs family G proteins have been shown to close K^+ channels (probably *via* a cAMP-dependent mechanism) (Madison and Nicoll, 1986; Pedarzani and Storm, 1995) and open cardiac L-type Ca^{2+} channels (mediated by a direct coupling of Gβγ subunits to the channel) (Fisher and Johnston, 1990). The hydrolysis of GTP to GDP by the dissociated Gαs subunit can be specifically blocked by ADP-ribosylation of the subunit by the bacterial cholera toxin from *Vibrio cholerae*. Cholera toxin transfers the ADP-ribose group from nicotinamide adenine dinucleotide (NAD^+) to an arginine residue at position 178 on the Gα subunit (Hsia *et al.*, 1985). This permanently activates the G protein and it cannot hydrolyse the bound GTP.

The Gi/o family comprises the $G\alpha i_{1\text{-}3}$, the brain specific $G\alpha o_{a\text{-}b}$, Gαz and the photoreceptor Gαt (transducin) G proteins (Morris and Malbon, 1999). The Gαi/o subunits act to inhibit adenylate cyclase and reduce the intracellular cAMP levels (Sullivan *et al.*, 1986; Yatani *et al.*, 1988). The Gi/o family act to open the G protein-coupled inwardly-rectifying K^+ channels (GIRK) and close neuronal N-type Ca^{2+} channels by direct Gβγ coupling (Logothetis *et al.*, 1987; Penington *et al.*, 1993; Krapivinsky *et al.*, 1995; Oh *et al.*, 1995; Herlitze *et al.*, 1996; Ikeda, 1996). Transducin (Gαt) acts to activate cGMP phosphodiesterase, which is the main effector mechanism in photoreceptor transduction (Morris and Malbon, 1999). With the exception of Gz (about which very little is known),

Gαi/o subunits can be specifically inhibited by the action of pertussis toxin from *Bordatella pertussis*, which transfers the ADP-ribose group from NAD^+ to a cysteine residue at position 350 on the Gα subunit to prevent the GDP/GTP exchange upon G protein activation (Hsia *et al.*, 1985).

The pertussis toxin insensitive Gq/11 family is comprised of the Gαq, Gα11 and Gα14-16 G proteins (Morris and Malbon, 1999). They can activate the beta isozymes of phospholipase C (PLCβ1–4), which hydrolyse phosphatidylinositol bisphosphate (PIP_2) to form the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 activates intracellular ionotropic IP_3 receptors on the endoplasmic reticulum to release the intracellular store of Ca^{2+} (Mak *et al.*, 1998). DAG directly activates protein kinase C, which phosphorylates many intracellular targets and can activate phospholipase D (Merritt *et al.*, 1986; Gierschik and Jakobs, 1987; Taylor *et al.*, 1991; Hammond *et al.*, 1997). Furthermore, the Gq/11 G protein family has been shown to modulate a novel K^+ current (Shi *et al.*, 2004) in addition to inhibiting the established GIRK current (Hill and Peralta, 2001).

The G12/13 family (G12 and G13) has been shown to couple to small G proteins *via* interactions with the Rho guanine nucleotide exchange factor (p115RhoGEF), a GTPase activating protein (GAP) for the small G protein Rho (Jiang *et al.*, 1998; Kozasa *et al.*, 1998). G12 G proteins can couple to (and activate) PLD, which may be dependent on Rho activity (Rumenapp *et al.*, 2001). They interact with the cytoskeletal proteins HSP90 and radixin (Vaiskunaite *et al.*, 2000; Waheed and Jones, 2002) and they have been shown to activate the non-receptor Bruton's tyrosine kinase (Jiang *et al.*, 1998; Kurose, 2003).

GPCR–mediated activation of heterotrimeric G proteins

One popular (and probably oversimplified) model for GPCR mediated stimulation is that an agonist binds to the receptor, within the pocket formed by the transmembrane domain (rhodopsin-like) or at the extracellular amino terminal domain (for other family GPCRs) (Dixon *et al.*, 1987; Strader *et al.*, 1987; Matsui *et al.*, 1989; Strader *et al.*, 1989b). The binding of agonist appears to elicit a conformational change within the receptor, probably involving movement of the second, third or sixth transmembrane helices relative to the other helices within the bundle (Farahbakhsh *et al.*, 1995; Karnik *et al.*, 2003; Miura *et al.*, 2003). Prevention of the helical movement in rhodopsin by artificial disulphide linkage and Zn^{2+} cross linkage has been demonstrated to inhibit activation of the receptor (Farrens *et al.*, 1996; Sheikh *et al.*, 1996). The helix movement is thought to involve an opening and unmasking of important residues on the intracellular surface, probably at the base of the third, sixth or seventh transmembrane helices (Abdulaev and Ridge, 1998; Ballesteros *et al.*, 2001). This allows the amino-terminal helix and carboxy-terminal domains of the Gα protein subunit (Blahos *et al.*, 2001), to bind the agonist occupied activated receptor at the third intracellular loop or tail domains (Limbird *et al.*, 1980; Strader *et al.*, 1989b; Wu *et al.*, 1998; El Far *et al.*, 2001). The Gα subunit of the G protein undergoes a conformational change, probably involving the switch region or a destabilisation of the amino-terminal helix (Lambright *et al.*, 1996; Remmers *et al.*, 1999; Yeagle and Albert, 2003) and the GDP that is bound to the trimer is exchanged for GTP. This GTP exchange in the switch region (which contacts the Gβγ subunits) causes the G protein to dissociate into the α and βγ forms that couple to intracellular effectors to activate or modulate signalling pathways (Limbird *et al.*, 1980; Stadel *et al.*, 1981; Logothetis *et al.*, 1987). The α-subunit, which has an intrinsic GTPase activity, hydrolyses GTP back to GDP, the G protein is inactivated and it re-associates (Bohm *et al.*, 1997; Strange, 1999). This simple view of the transient activation of the heterotrimeric G protein by GPCRs has been complicated somewhat by the demonstration that heterotrimeric G proteins can bind unliganded GPCRs and that

intracellular effectors can be co-immunoprecipitated with GPCRs as a complex (Chidiac, 1998; Lachance *et al.*, 1999). It is apparent, therefore, that the activation mechanism of G proteins by GPCRs is a subtle and complex process that deserves continuing attention.

The M_3 muscarinic G protein-coupled receptor

The parasympathetic nervous system utilises the endogenous neurotransmitter acetylcholine to elicit various effects in the central nervous system and peripheral tissues. Examples of parasympathetic stimulation include a reduction in heart rate and force of contraction, the stimulation of gastric acid and glandular secretion, the contraction of visceral smooth muscle, the contraction of the ciliary body within the eye and an indirect relaxation of vascular smooth muscle tone (due to the co-transmission of nitric oxide) (Kayaalp and Turker, 1969; Carrow *et al.*, 1975; Kowalewski *et al.*, 1975; Lonnerholm and Widerlov, 1975; Sarna and Daniel, 1975).

The physiological effects of the muscarinic agonist muscarine (from the mushroom *Amanita muscaria*) and antagonist atropine (from the Deadly Nightshade *Atropa belladonna*) have been recognised for many years (Ford, 1909; Dale, 1914). More recent pharmacological studies on the mechanism of muscarinic receptor signalling indicated that there were a number of different subtypes within the family, as the inhibition of adenylate cyclase and the hydrolysis of phosphoinositides, coupled with different ligand dissociation constants, were observed in the activation of various muscarinic-type receptors (Haga *et al.*, 1985; Kubo *et al.*, 1986; Bonner *et al.*, 1987; Fukuda *et al.*, 1987; Peralta *et al.*, 1987a). The muscarinic receptor family was subsequently determined to comprise of five subtypes, which were named according to their chronological characterisation (Kubo *et al.*, 1986; Bonner *et al.*, 1987; Bonner, 1989). The M_1, M_3 and M_5 receptors all couple to Gq/11 family G proteins and elicit activation of phospholipase C leading to downstream activation of protein kinase C (PKC) and an increase in intracellular Ca^{2+} concentration (Jones and Michell, 1974; Bonner, 1989; Buckley *et al.*, 1989). The M_2 and M_4 receptors couple

through Gi/o family G proteins to inhibit adenylate cylase (Haga *et al.*, 1985; Bonner, 1989). They also elicit an inward rectifying K^+ current, activated by the Gβγ subunits (Bunemann and Hosey, 2001). The M_3 receptor can also signal to activate phospholipase D *via* an additional pertussis toxin insensitive G12 activation in some cell types, rather than the Gq dependent PKC-mediated PLD activation (Singer *et al.*, 1996; Mitchell *et al.*, 1998; Rumenapp *et al.*, 2001).

The M_3 muscarinic G protein-coupled receptor was first cloned in 1987 (Bonner *et al.*, 1987; Peralta *et al.*, 1987a). It is found widely distributed in neuronal cells and in the central nervous system (CNS), as well as in the peripheral ganglia, in visceral and vascular smooth muscle, exocrine glands and the ciliary body of the eye (Doods *et al.*, 1994; Levey *et al.*, 1994; de la Vega *et al.*, 1996; Zhang, 1996; Hoglund and Baghdoyan, 1997; Lau and Pennefather, 1998; Masuda *et al.*, 1998). The human M_3 receptor is 590 amino acids in length (approximately 66 kDa in size) and, like most rhodopsin-like GPCRs, contains a number of N-linked glycosylation sites on asparagine residues at positions 5, 6, 15 and 41 in the extracellular amino-terminal domain. Cysteine residues at positions 141 and 221 form the disulphide bridge present in nearly all GPCRs and the receptor is palmitoylated on cysteine residues 561 and 563 in the intracellular carboxy-terminal tail domain (Peralta *et al.*, 1987b; Bonner, 1989). Mutagenesis and modelling studies have demonstrated that the acetylcholine ligand binding site is probably formed by multiple tyrosine and threonine residues within the transmembrane domain α-helices (TM), which coordinate to provide a binding pocket on the internal face of the α-helix barrel. This ligand binding domain involves the conserved Asp-148, which coordinates the protonated nitrogen of acetyl-choline, Tyr-149 in TM3, Thr-232 and Thr-235 in TM5, Tyr-507 in TM6 and Tyr-530 and Tyr-534 in TM7, mutations of which were not shown to appreciably affect receptor expression or G protein coupling, but reduced the agonist binding affinity (Wess *et al.*, 1991; Wess, 1993). A proline residue (201) in TM4, which does not appear to interact with the ligand, is also thought to be necessary for binding (Wess *et al.*, 1991; Wess, 1993). The

M_3 receptor has an unusually long third intracellular loop (i3) of approximately 239 amino acids (R253-Q491). The heterotrimeric G protein subunit Gβγ interacts at a domain determined to be in the i3 region at residues 289-330 and the phosphorylation by G protein-coupled receptor kinase (GRK) putatively occurs at residues 331-333 and 348-352 (Wu *et al.*, 2000), although this phosphorylation may not be necessary for internalisation (Shockley *et al.*, 1999; Budd *et al.*, 2000). The small G protein ADP-ribosylation factor (ARF) also associates with the muscarinic receptor at the third intracellular loop (Robertson *et al.*, 2001; Mitchell *et al.*, 2003).

Receptor desensitisation and endocytosis

The stimulation of intracellular effectors by G protein-coupled receptors can often be measured within seconds (Yang *et al.*, 1967; Mukherjee *et al.*, 1975; Cockcroft, 1984). So that the activation of effectors by the receptors is attenuated despite the continued presence of agonist, a number of negative feedback mechanisms exist to 'switch off' the signal provided by the activation of the GPCR and G protein.

One of the most important mechanisms by which the receptor signalling is attenuated is by the agonist-dependent uncoupling of the G protein (desensitisation) and subsequent endocytosis of the receptor away from the plasma membrane (internalisation or sequestration). This process often begins with the specific phosphorylation of the receptor, at a site rich in serine and threonine residues, often within the carboxy-terminal tail domain or the third intracellular loop on most GPCR types (Dohlman *et al.*, 1987; Leeb-Lundberg *et al.*, 1987; Krupnick and Benovic, 1998). This phosphorylation can be mediated by a member of the G protein receptor kinase (GRK) family in response to agonist stimulation of the receptor (Kuhn, 1974; Sibley *et al.*, 1984; Bunemann and Hosey, 1999). In the case of the M_3 receptor, the GRK that is involved may be GRK6 (Willets *et al.*, 2003) or GRK2 (Wu *et al.*, 1998). Some GRKs may be activated by the Gβγ subunits of the heterotrimeric G protein as a consequence of agonist activation (Haga *et al.*, 1994) and some

phosphorylate the receptor in the presence of PIP_2 (Pitcher *et al.*, 1995; Willars *et al.*, 1996). Because the phosphorylation by GRKs only affects the activated receptor, the term homologous desensitisation has been applied to this process (Sibley *et al.*, 1985). Other effector kinases such as casein kinase 1α (Tobin *et al.*, 1997; Budd *et al.*, 2000) and protein kinases A and C (Benovic *et al.*, 1985; Bouvier *et al.*, 1987; Hausdorff *et al.*, 1989) can also mediate phosphorylation of the GPCR and interrupt signalling to G proteins. In this latter instance, the receptor does not necessarily have to be agonist occupied and phosphorylation of other non activated (and different liganded) receptors also takes place - the term heterologous desensitisation has been applied to this type of mechanism.

Homologous desensitisation of the receptor, following GRK phosphorylation, often involves the recruitment of proteins known as arrestins that act to uncouple the G protein from the receptor by binding to regions on the receptor to occlude them from G protein interaction (Wu *et al.*, 1997; Krupnick and Benovic, 1998; Wu *et al.*, 2000). There are four types of known arrestin (Freedman and Lefkowitz, 1996); visual arrestin and cone arrestin are localised to the visual system (Shinohara *et al.*, 1987; Yamaki *et al.*, 1987; Murakami *et al.*, 1993; Craft *et al.*, 1994), β-arrestin 1 (or arrestin-2) and β-arrestin 2 (or arrestin-3) are expressed ubiquitously and act as adapter proteins for the clathrin-mediated endocytosis of many GPCRs (Lohse *et al.*, 1990; Attramadal *et al.*, 1992; Goodman *et al.*, 1996; Lin *et al.*, 1997). The carboxy-terminal region of the β-arrestins (residues 371-377 in β-arrestin 2), but not the visual arrestins, can bind to the amino-terminal domain of the clathrin heavy chain (Goodman *et al.*, 1996; Krupnick *et al.*, 1997a; Krupnick *et al.*, 1997b) and can additionally bind the AP-2 clathrin adapter complex to facilitate the clathrin coated pit-mediated endocytosis of GPCRs (Laporte *et al.*, 1999; Laporte *et al.*, 2000). In addition, the large GTPase, dynamin, which is involved in vesicle budding from the plasma membrane, may facilitate receptor endocytosis for different GPCR types (Ahn *et al.*, 1999; Claing *et al.*, 2002). Interestingly, some recent studies have suggested that phospholipase D (PLD) activity may also be implicated in the endocytosis of certain G protein-coupled receptors.

The PLD2 isoform has been proposed to be important for the endocytosis of both the angiotensin II type 1 GPCR (Du *et al.*, 2004) and the μ-opioid GPCR (Koch *et al.*, 2003; Koch *et al.*, 2004). This process may be mediated by phosphatidic acid (the product of PLD activation) as PA has been shown to be important in other membrane trafficking events and may influence the physical characteristics of the phospholipid membrane to allow efficient vesicle budding (Andresen *et al.*, 2002). Following sequestration, the receptors are processed by early endosomes where the ligand is dissociated and the receptor is dephosphorylated and recycled back to the plasma membrane swiftly (resensitised). Alternatively the receptor is held in a subcellular pool or transported to lysosomes for degradation (Claing *et al.*, 2002). The particular fate of the GPCRs may be dependent on specific serine residues within the carboxy-terminal tail domains of the receptors, which appear to modulate the recycling kinetics of the receptors (Innamorati *et al.*, 1998; Oakley *et al.*, 1999; von Zastrow, 2003).

Another of the mechanisms that modulate GPCR signalling is the regulation of heterotrimeric G protein activity by the regulator of G protein signalling (RGS) proteins. These are cofactors that belong to the family of GTPase-activating proteins (GAPs) that act to accelerate hydrolysis of GTP back to GDP by G proteins; RGS proteins act specifically on the Gα subunit of the heterotrimeric G protein to drive the G protein (and subsequently the GPCR) back into an inactive conformation (Berman and Gilman, 1998). This is distinct from desensitisation, as the mechanism effectively acts to recycle the signalling properties provided by the receptor faster than the receptor/G protein complex could under normal conditions. The RGS family consists of more than twenty proteins (some with splice variants), which range in size from 159 amino acids (~19 kDa) (RGS13) to 1447 amino acids (~156 kDa) (RGS12) (Chatterjee and Fisher, 2000; Strausberg *et al.*, 2002). All RGS proteins have a conserved domain (the RGS domain) of 117 residues and many have other homologous domains including a PDZ domain (RGS12), a G protein gamma-like subunit (GGL) domain (RGS6, RGS7, RGS9 and RGS11) and a Rap1/2 binding (RBD) domain

(RGS12 and RGS14) (Hollinger and Hepler, 2002). The RGS proteins directly bind, with high affinity, the GTP-bound Gα subunit of the heterotrimeric G protein and overexpression of RGS4, for example, has been shown to inhibit both the Gi/o and Gq coupled signalling in mammalian cells (Druey *et al.*, 1996). However, at lower physiological levels, RGS4 has been shown to interact transiently with Gq, to facilitate an oscillation of Ca^{2+} release within the cell (Luo *et al.*, 2001; Hollinger and Hepler, 2002). RGS proteins may additionally function as molecular switches, which may modulate the signalling characteristics of a G protein. Rumenapp and colleagues demonstrated that overexpression of RGS4 (which is specific for Gq) could inhibit M_3 receptor-activated Gq-mediated PLC activation, without affecting G12/13-mediated PLD responses, whilst expression of the RGS homology domain of Lsc (which is specific for G12/13) could specifically inhibit PLD but not PLC responses (Rumenapp *et al.*, 2001). RGS proteins may therefore be important and useful targets for elucidating important GPCR mediated signalling pathways.

Signal crosstalk and convergence

The signalling by G protein-coupled receptors is complicated and diverse. The simple notion of a single GPCR coupling to a single specific type of G protein to activate a single type of downstream effector is becoming increasingly inaccurate. Many hundreds of different types of GPCRs, many with vastly different sequences exist in mammals and these all couple through a similar subset of G proteins to regulate effectors. There is increasing evidence that many of the receptor/G protein pathways can amplify and influence other separate but parallel signalling pathways. This is known as signal crosstalk and can occur at various levels of the signalling cascade.

At the level of receptor/G protein signalling, there are many reports suggesting that various heterotrimeric G proteins can influence and even regulate the signals from another GPCR and G protein. An example of this is the agonist stimulated Gi/o coupled δ-opioid receptor synergistically potentiating the Gq coupled M_3 muscarinic receptor activated intra-

cellular Ca^{2+} release in SH-SY5Y cells (Yeo *et al.*, 2001). This Gi/o enhancement of Gq modulated signalling is repeated in many other findings (Megson *et al.*, 1995; Selbie *et al.*, 1995; Dickenson and Hill, 1997) and is probably due to the Gβγ subunit interacting directly with PLCβ (Selbie *et al.*, 1997). In addition, stimulation of protein kinase C by phorbol esters has been shown to potentiate the accumulation of cAMP in cells by Gs stimulation (which may be dependent on the isoforms of adenylate cyclase involved) (Karbon *et al.*, 1986).

It has also been shown that GPCRs can modulate the signalling of other GPCR/G protein independent signalling pathways and vice-versa. The β-adrenergic receptor has been shown to be phosphorylated *in vitro* and *in vivo* by the insulin and insulin-like growth factor tyrosine kinase receptors and although no functional consequences were observed, the implication was that the adrenergic receptors may be heterologously desensitised and cease signalling (Baltensperger *et al.*, 1996; Karoor and Malbon, 1996). It has been demonstrated that G protein Gβγ subunits can mediate the signalling to the MAPK pathway of the insulin-like growth factor tyrosine kinase receptor (Luttrell *et al.*, 1995) and Gβγ subunits have been shown to directly associate with c-Raf in a yeast two-hybrid assay and *in vitro* (Pumiglia *et al.*, 1995), however any potential functional role of this association was not addressed. Many GPCRs have been shown to activate the MAP kinase pathway and the phosphorylation, arrestin recruitment and internalisation of GPCRs have all been implicated in the activation of MAPK to some extent, depending on the receptor type (Hawes *et al.*, 1995; Pierce *et al.*, 2000). The heterologous phosphorylation of the β-adrenergic receptor by protein kinase A leads to an arrestin mediated recruitment of c-Src to the receptor and subsequent endocytosis-dependent activation of the MAPK pathway (Daaka *et al.*, 1997; Luttrell *et al.*, 1999). The similar activation of MAPK by the M_1 muscarinic, formyl peptide and the κ-, δ- and μ-opioid receptors has been shown to be dependent upon receptor endocytosis (Ignatova *et al.*, 1999; Vogler *et al.*, 1999; He *et al.*, 2001). However, the

necessity of GPCR internalisation has been disputed in the case of the opioid receptors, as agonist activation has also been shown to activate MAPK without endocytosis (Kramer and Simon, 2000). In addition, the heterologous phosphorylation of the M_3 muscarinic receptor by PKC can activate the MAPK pathway without involving receptor endocytosis (Budd *et al.*, 1999; Budd *et al.*, 2001) and internalisation-independent MAPK activation by other GPCRs such as the α_2-adrenergic and cannabinoid receptors has been demonstrated (DeGraff *et al.*, 1999; Roche *et al.*, 1999; Schramm and Limbird, 1999). The functional significance of receptor internalisation for the activation of the MAPK pathway remains elusive, however the evidence remains that co-activation of other signalling pathways by GPCRs is an important mechanism of signal transduction.

Key signalling pathways of the M_3 receptor: Phospholipase C

A novel enzymatic activity was described in the 1950s, which resulted in the incorporation of ^{32}P into phospholipids upon carbachol stimulation of pigeon pancreatic slices (Hokin and Hokin, 1953). Subsequent work done by Michell and colleagues, resulted in the characterisation of a membrane-associated enzyme, phospholipase C. This enzyme was shown to catalyse the formation of 1,2-diacylglycerol and inositol phosphate from the membrane lipid phosphatidylinositol, which was intriguingly coupled to a concomitant increase in intracellular Ca^{2+} levels (Lapetina and Michell, 1972; Lapetina and Michell, 1973a; Lapetina and Michell, 1973b; Jones and Michell, 1974; Allan and Michell, 1975; Akhtar and Abdel-Latif, 1980).

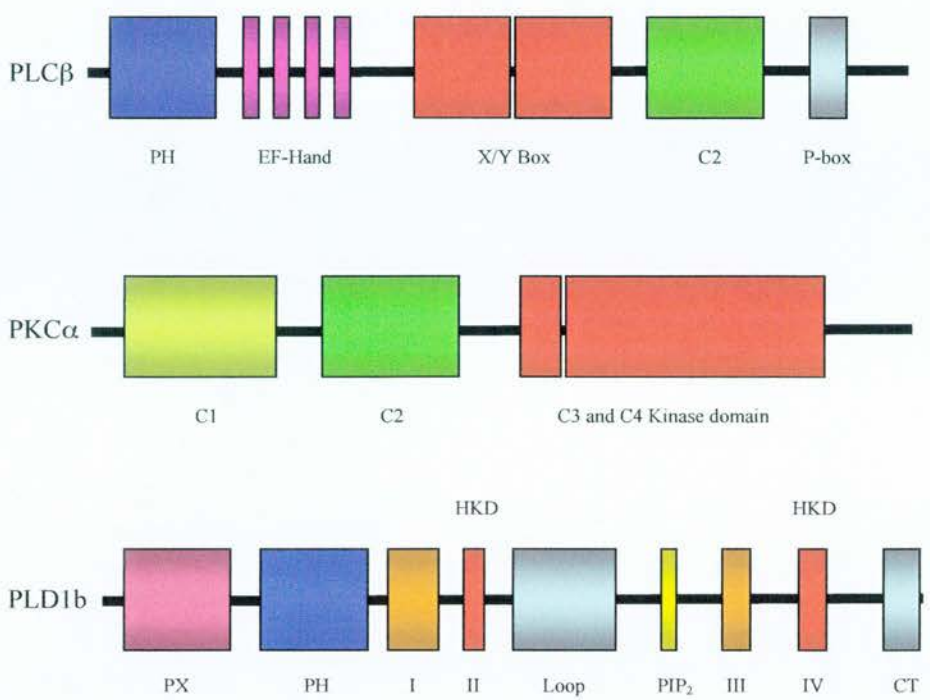
Mammalian PLC isoforms are generally phosphatidylinositol specific (PI-PLC) and preferentially hydrolyse the phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2) into sn 1,2-diacylglycerol (DAG) and myo-inositol (1,4,5)-trisphosphate (IP_3) (Griffin and Hawthorne, 1978; Ryu *et al.*, 1987; Berridge, 1993). DAG and IP_3 then mediate the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular reserves respectively (Streb *et al.*, 1983; Nishizuka, 1984; Putney *et al.*, 1986). There are eleven

mammalian isoforms of PLC, grouped into four main PLC families; there are four subtypes of PLCβ (~145-155 kDa), two subtypes of PLCγ (~145-155 kDa) four of PLCδ (~85 kDa) and a novel PLCε isoform (~86 kDa) (Suh *et al.*, 1988; Thomas *et al.*, 1991; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Berridge, 1993; Rhee and Bae, 1997; Wing *et al.*, 2003). PLC activity in response to agonist stimulated GPCRs was determined to act *via* a heterotrimeric G protein mechanism by Taylor and Merritt (Merritt *et al.*, 1986) and subsequent analysis revealed that the PLC activity was due to the PLCβ isoform (Blank *et al.*, 1991; Taylor *et al.*, 1991; Shaw and Exton, 1992; Exton, 1993). PLCβ isoforms are activated by the Gq family of heterotrimeric G proteins (Blank *et al.*, 1991; Taylor *et al.*, 1991; Lee *et al.*, 1992; Wu *et al.*, 1992). Purified PLCβ1 is more potently activated than PLCβ2 and PLCβ3 (which showed little difference in activation) by purified Gαq and Gα11 subunits (Hepler *et al.*, 1993; Smrcka and Sternweis, 1993). It has been determined that both the Gα and Gβγ subunits can activate members of the PLCβ family with different potencies - the activation of PLC by the Gβγ subunits is of approximately 50-100 fold lower potency than that by the Gα subunits (Morris and Scarlata, 1997). However, as the PLCβ isoform has a GTPase activating protein (GAP) effect for the Gα subunit (Berstein *et al.*, 1992; Paulssen *et al.*, 1996), the use of the non-hydrolysable GTP analogue (GTPγS) would negate the GAP effect of PLC and may account for the higher potency of Gα activation *in vitro* (Morris and Scarlata, 1997). The potency of PLCβ activation by Gβγ subunits is also isoform dependent, with PLCβ2 and PLCβ3 being more sensitive than PLCβ1 and PLCβ4 (Smrcka and Sternweis, 1993; Ueda *et al.*, 1994).

PLCβ is the only isoform subfamily that is directly activated by the Gq/11 hetero-trimeric G protein family (Rhee and Choi, 1992) and this is thought to be due to PLCβ having an extended carboxy-terminal domain compared to PLCδ and PLCγ (Lee *et al.*, 1992; Park *et al.*, 1993). Truncations of this tail portion abolished the enzymes ability to be activated by Gαq (although not by Gβγ) (Park *et al.*, 1993; Wu *et al.*, 1993) suggesting that

this region contains a Gαq binding site. The Gβγ binding site is thought to be in the N-terminal pleckstrin homology (PH) domain, necessary for phosphatidylinositol bisphosphate (PIP_2) binding (Kuang *et al.*, 1996). There are recent reports that PLCε can be activated in response to G protein-coupled receptor stimulation, which could be mediated by the Gα12/13 heterotrimeric G protein family (Wing *et al.*, 2003; Kelley *et al.*, 2004) and which may also be mediated by an elevated level of intracellular cAMP (Evellin *et al.*, 2002). The PLC family have a common main structure consisting of an amino-terminal pleckstrin homology (PH) domain, an EF hand domain that acts as a flexible linking domain to the conserved catalytic core of the enzyme (the X/Y box) and a C2 region at the carboxy-terminal of the protein (Figure 1.3) (Rhee and Choi, 1992). The PH domain is approximately 120 amino acid residues long and is necessary for the association of PLC with phospholipid head groups in the membrane and specifically with PIP_2 (Paterson *et al.*, 1995; Lomasney *et al.*, 1996; Yagisawa *et al.*, 1998). The conserved catalytic core of the enzyme begins at approximately residue 300 and consists of two parts - the X region is approximately 147 residues and is connected to the Y region, which is approximately 118 residues in length. In PLCβ and PLCδ, this X/Y box is separated by 50-70 amino acids, however PLCγ has over 400 residues between the two regions, containing three src-homology (SH) domains (two SH2 domains and one SH3 domain) (Rhee and Choi, 1992; Essen *et al.*, 1996). The catalytic domain contains residues that are important for phospho-inositol hydrolysis (Lys-438, Lys-440, Ser-522 and Arg-549 in PLCδ) and also other residues that are important for catalysis (His-311 in PLCδ) (Ellis *et al.*, 1998). The C2 domain is similar to that found in the protein kinase C superfamily (where it is necessary for allosterically binding Ca^{2+} ions for activity), however it may primarily function as a stabilising domain, as mutations to prevent Ca^{2+} binding do not significantly alter the activity of the enzyme (James and Downes, 1997). The carboxy-terminal domain of the enzyme, in addition to binding the Gαq subunit, has an intrinsic GTPase activating protein-

Figure 1.3



The domain structure of the phospholipases C and D, and protein kinase C.

The domain structures of the major GPCR pathway-coupled isozymes of PLC, PKC and PLD. The catalytic domains in each are indicated in red. The pleckstrin homology (PH) domains in PLC and PLD (blue) are important for associating with lipid membranes. The EF-hand and C2 regions in PLC and C2 region in PKC are important for Ca^{2+} ion binding. The P-box in PLCβ is important for interaction with the heterotrimeric G proteins. PKC contains an additional conserved C1 domain, where diacylglycerol and phorbol esters bind. PLD contains a phox homology (PX) domain and the conserved regions I-IV include the catalytic HKD domains. PLD contains an additional loop region, an allosteric PIP_2 site and the carboxy-terminal tail domain (CT) is important for regulating activity of the enzyme.

like (GAP-like) activity, which accelerates hydrolysis of the GTP on the active Gαq subunit back to the inactive GDP form (Paulssen *et al.*, 1996).

Key signalling pathways of the M_3 receptor: Phospholipase D

Phospholipase D (PLD) activity was originally described in plants (Hanahan and Chaikoff, 1947). Originally thought to be present only in simple eukaryotes such as *Dictyostelium discoideum* (Ellingson and Dischinger, 1984; Cubitt *et al.*, 1993) and plants (Dawson, 1967; Long *et al.*, 1967a), it was characterised in mammals only in the 1970s (Saito and Kanfer, 1973). PLD uniquely hydrolyses phospholipids at the terminal phosphodiester bond to produce phosphatidic acid (PA) and the free polar head group. The preferred substrate for PLD, phosphatidylcholine (PtdCho), is the most abundant phospholipid constituent of mammalian membranes, providing around 50% of the total phospholipid content and as much as 60% of the intracellular membrane phospholipid content (Owen *et al.*, 1981; Lagarde *et al.*, 1982; Patton *et al.*, 1982). The phosphatidyl transferase ability of PLD has been utilised as an unequivocal indication of PLD activity (Dawson, 1967; Long *et al.*, 1967b; Long *et al.*, 1967a; Yang *et al.*, 1967). In the presence of a primary alcohol such as butan-1-ol, activated PLD transfers the phosphatidyl moiety of the phospholipid to the alcohol and produces phosphatidylbutanol. The phosphatidylbutanol product is not metabolised further by the action of phosphatidic acid phosphohydrolase (see below). Thus PLD activity can be assayed by monitoring the production of [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) when [^{3}H]palmitate labelled cells are stimulated in the presence of 0.1-0.5% (v/v) butan-1-ol. This transphosphatidylation assay is a specific way of measuring phospholipase D activity within whole cells and a scheme is shown in Figure 1.4.

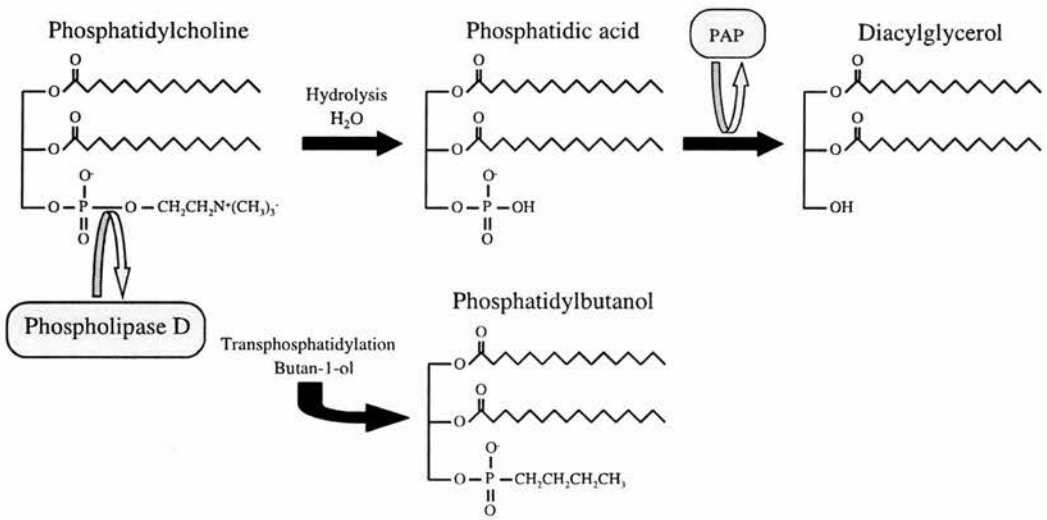
The function of PLD activity has remained somewhat elusive; it was originally postulated that the release of the choline group by PLD is an essential step in the synthesis of the neurotransmitter acetylcholine (Hattori and Kanfer, 1984; Blusztajn *et al.*, 1987; Zhao *et al.*, 2001) and whilst this may be the case in many neuronal cells, the currently favoured

hypothesis is that the PA group released during hydrolysis may function as an additional second messenger in peripheral tissues (Exton, 1990; English, 1996).

Phosphatidic acid (PA) has been implicated in a variety of cellular functions and includes possible calcium mobilisation independent to that caused by the action of IP_3 (English, 1996), cellular proliferation (Knauss *et al.*, 1990) and as a substrate for downstream prostaglandin synthesis (Marshall *et al.*, 1981). It has also been implicated in the activation of certain kinases (Ohguchi *et al.*, 1997). Furthermore, phosphatidic acid has been shown to have a critical role in activation of the regulatory kinase mTOR (target of rapamycin), a phosphatidylinositol 3-kinase related kinase (PIKK), which is a potentially oncogenic protein that is involved in cell cycle regulation and proliferation (Fang *et al.*, 2001). Moreover, phosphatidic acid is an important regulator of the mitogen activated protein kinase (MAPK) signalling mechanism (Ghosh *et al.*, 1996; Rizzo *et al.*, 1999). PA appears to directly bind to the serine-threonine kinase c-Raf (residues 389-423) to facilitate 14-3-3-mediated translocation of c-Raf to the plasma membrane *in vivo* and is possibly a cofactor for full activation of c-Raf (Ghosh *et al.*, 1996; Rizzo *et al.*, 1999; Andresen *et al.*, 2002; Ghosh *et al.*, 2003).

Phosphatidic acid is further metabolised by phosphatidic acid phosphohydrolase (PAP) to produce diacylglycerol (DAG) (Brown *et al.*, 1990; Sciorra and Morris, 1999). A second period of elevation of DAG following GPCR activation is thought to be more sustained than the original PLC-mediated release and probably arises due to the rate limiting action of PAP on phosphatidic acid (Martinson *et al.*, 1990). Initial reports indicated that the DAG derived from PAP action on PA was not efficient at activating PKC (Martin *et al.*, 1989; Kiley *et al.*, 1991), however, more recent reports have indicated that DAG derived from PA is effective at activating PKC isoforms (although it may be more effective at activating Ca^{2+}-independent PKC isoforms) (Ha and Exton, 1993b).

Figure 1.4



The unique phospholipase D catalysed transphosphatidylation reaction.

The *in vivo* scheme of PLD activity is shown (top), with PLD hydrolysing the terminal phosphodiester head group of phosphatidylcholine (PtdCho) to produce phosphatidic acid (PA) and choline (not shown, for clarity). The PA is then further metabolised to diacylglycerol (DAG) by the action of phosphatidic acid phosphohydrolase (PAP). PLD has a higher affinity for primary alcohols than H_2O, so in the presence of a primary alcohol, the catalysis proceeds with the alcohol as the substrate (bottom). This results in an accumulation of phosphatidyl-alcohol that cannot be metabolised further. The phosphatidyl-alcohol levels can be assayed if the fatty acid moiety of phosphatidylcholine has been radiolabelled.

PA can also be metabolised to lysophosphatidic acid (lysoPA) by the actions of phospholipase A_1 or A_2 (Gerrard and Robinson, 1989; Gaits *et al.*, 1997). LysoPA is bioactive and it has also been shown to mobilise Ca^{2+}, activate PKC and inhibit cAMP formation by acting on Edg family G protein coupled receptors (van Corven *et al.*, 1989; Jalink *et al.*, 1990; van der Bend *et al.*, 1992; Hecht *et al.*, 1996; An *et al.*, 1997; Bandoh *et al.*, 1999). It has been shown to display the properties of a growth factor and causes cellular proliferation (van Corven *et al.*, 1989; Moolenaar *et al.*, 1992). Following the release of lysoPA from activated platelets, which cause aggregation, there are high levels of lysoPA found in serum (Eichholtz *et al.*, 1993; Sano *et al.*, 2002). LysoPA has also been shown to be important in the activation of the PKCμ (PKD) isoform (Kam and Exton, 2004).

PLD has additionally been proposed to be important in mediating anti-apoptotic signalling in a number of different cell types - glutamate induced cell death in PC12 cells was attenuated by overexpression of PLD isoforms (Kim *et al.*, 2003) and an increased PLD activity could protect against hydrogen peroxide (H_2O_2) and hypoxia induced cell death in PC12 cells (Lee *et al.*, 2000a; Yamakawa *et al.*, 2000). Furthermore, elevated PLD activity could also protect against apoptosis in human neutrophils and vascular smooth muscle cells with the protection possibly being mediated by phosphatidic acid and microtubule stabilisation (Park *et al.*, 2002; Zheng *et al.*, 2004). There are conflicting reports from some groups however, suggesting that enhanced PLD activity can sensitise a cell to promote apoptosis rather than protect it (Kang *et al.*, 1998; Zhong *et al.*, 2002). The role of PLD in the apoptotic pathway remains ambiguous, nevertheless it may have important implications for cellular survival.

PLD isoforms

The two mammalian phosphatidylcholine specific phospholipase D isoforms, the 124 kDa PLD1 and 106 kDa PLD2 were cloned in 1995 and 1997 (Hammond *et al.*, 1995; Colley *et al.*, 1997b; Kodaki and Yamashita, 1997; Lopez *et al.*, 1998). PLD1b, a splice variant of PLD1 lacking the 38 internal amino acids 565-624, has been cloned and is apparently regulated in the same way as PLD1a (Hammond *et al.*, 1997). Other PLD activities distinct from these isoforms have also been described, such as the phosphatidylinositol-glycan specific PLD isolated from human and bovine sera (Davitz *et al.*, 1987; Scallon *et al.*, 1991) and a phosphatidylcholine specific PLD activity isolated in porcine lung, which had a molecular mass of 190 kDa (Okamura and Yamashita, 1994). This oleate-sensitive PLD activity has been proposed as being due to a novel isozyme (Kawabe *et al.*, 1998; Lee *et al.*, 1998), however other groups believe it is likely to be due to PLD2 activity (Kim *et al.*, 1999b; Sarri *et al.*, 2003)

Protein Kinase C stimulated PLD activity

The activation of PLD as a consequence of GPCR agonist-stimulated PLC activity, leading to downstream activation of protein kinase C (PKC) by diacylglycerol (DAG) (a product of the PLC mediated hydrolysis of PIP_2) has been recognised for some time (Cockcroft, 1984; Martinson *et al.*, 1989; Brown *et al.*, 1990). The tumour-promoting phorbol esters, which activate protein kinase C (Castagna *et al.*, 1982; Nishizuka, 1984), were known to indirectly stimulate PLD activity and therefore implicated PKC in the direct activation of PLD (Billah *et al.*, 1989; Gelas *et al.*, 1989; Huang and Cabot, 1990). In 1992 Conricode and colleagues demonstrated that PKC could indeed stimulate PLD activity (Conricode *et al.*, 1992) and in 1996, recombinant PKCα and purified PKCα from porcine brain were shown to interact with and activate PLD1 synergistically with ARF and in a kinase-independent manner (Singer *et al.*, 1996). Moreover, the conventional PKCβ isoform, but not the PKCγ isoform, was shown to stimulate PLD activity, but with a lower potency than PKCα, whilst PKCδ,

PKCε and PKCζ failed to stimulate PLD activity (Conricode *et al.*, 1994). However, the use of PKC inhibitors, such as staurosporine and bisindoylmaleimide has yielded information that agonist-dependent GPCR-stimulated PLD activity is dependent on catalytic activation of PKC in many cell types (Cook *et al.*, 1991; Plevin *et al.*, 1994; Ahmed *et al.*, 1995; Martinson *et al.*, 1995; Meacci *et al.*, 1995; Yeo and Exton, 1995; Rumenapp *et al.*, 1997). It has been demonstrated that both PLD1 and PLD2 isozymes are activated by PKC isoforms with similar potencies and in a direct manner (Xie *et al.*, 2002; Chen and Exton, 2004). In PC12 cells, bradykinin and hydrogen peroxide stimulated PLD2 activity is potentially mediated by PKCδ and PKCε as well as by PKCα (Lee *et al.*, 2000b; Oh *et al.*, 2000).

The protein kinase C superfamily consists of five main subfamilies, each with different isozymes. The classical or conventional PKC (cPKC) subfamily consists of the isoforms PKCα, PKCβ and PKCγ. These three isoforms (the first to be cloned) were elucidated originally due to their dependence on Ca^{2+} for activation by diacylglycerol and their activation by the tumour promoting phorbol esters (Takai *et al.*, 1977; Takai *et al.*, 1979; Kikkawa *et al.*, 1982; Coussens *et al.*, 1986; Parker *et al.*, 1986). Soon three more PKC isoforms were discovered - PKCδ, PKCε and PKCζ (Ono *et al.*, 1987). The isoforms PKCδ and PKCε, together with PKCη (Osada *et al.*, 1990) and PKCθ (Osada *et al.*, 1992), make up the novel PKC subfamily (nPKC). These four PKCs are still activated by diacylglycerol, and are also stimulated by phorbol esters, however they lack the Ca^{2+} dependent C2 region found in the conventional PKCs, so are not dependent on Ca^{2+} for kinase activity (Osada *et al.*, 1990). The isoforms PKCζ and PKCι (Ono *et al.*, 1989b) make up the atypical PKCs (aPKC), which are both Ca^{2+} and DAG insensitive (Ono *et al.*, 1989b). There are also PKCν and PKCμ (or PKD) isoforms, which may comprise another PKC subfamily. These PKCs are insensitive to Ca^{2+}, DAG and are not activated by phorbol esters (Johannes *et al.*, 1994; Hayashi *et al.*, 1999). Three other gene products make up the protein

kinase C related kinase (PRK) subfamily, which consists of PRK1-3 (Mellor and Parker, 1998). These proteins are like the atypical PKCs in regulation, but can also bind the activated small G protein RhoA to increase their kinase activity (Mellor and Parker, 1998). With the exception of the PRK subfamily, all PKCs contain a conserved C1 domain region that contains one or two zinc finger motifs rich in cysteine and histidine residues (the C1 region is slightly altered in the atypical PKCs) (Mellor and Parker, 1998). The C1 domain is also the region responsible for binding of phorbol ester and phospholipids (Ono *et al.*, 1989a; Zhang *et al.*, 1995) and in the absence of activators acts as an autoinhibitory domain, which associates with the catalytic domain C4 to inactivate the enzyme (Orr *et al.*, 1992). The C2 region found in the conventional PKCs has homologous structure to the conserved region V_0 found in the other PKCs, however it is the only region that is dependent upon allosteric binding of Ca^{2+} ions for activity (due to a number of aspartate residues that are not present in the V_0 region) (Ono *et al.*, 1989a; Shao *et al.*, 1996; Mellor and Parker, 1998). The catalytically active kinase domain is conserved in all of the PKC superfamily and consists of two regions - C3 and C4. The C3 region is thought to coordinate ATP as the phosphate donor and C4 is thought to bind the substrate to be phosphorylated (Figure 1.3) (Ron and Kazanietz, 1999).

The region that is thought to interact with PLD is the amino-terminal regulatory domain of PKCα and this direct interaction-dependent activation of PLD may not be dependent upon the kinase activity of PKC (Singer *et al.*, 1996). Studies utilising truncations of the amino terminal of rat and human PLD1 yielded information that the region involved with PKCα activation was present in the first 325 residues of the PLD1 isozyme (within the PX domain – see below) (Park *et al.*, 1998; Sung *et al.*, 1999). Further studies revealed that the insertion of the residues GVPLE at position 87 of human PLD1 could lead to a loss of regulation by PKC (Zhang *et al.*, 1999b). This mutant was termed PIM87 PLD1.

Furthermore, the interaction of PKC with PLD isozymes has been shown to increase phosphorylation of PLD, which may negatively regulate PLD activity (Hu and Exton, 2003; Chen and Exton, 2004). In addition to stimulation of PLD by the conventional PKCα, recent reports have demonstrated a negative regulation of PLD1 by the competitive association of the novel PKCδ with PLD1. There is even evidence to suggest that PLD is basally negatively regulated by PKCδ *in vivo*, as cells containing both PKCα and PKCδ isoforms elicited a lower PLD response and attenuated PKCα and PLD association compared to those containing PKCα alone (Hornia *et al.*, 1999; Oka *et al.*, 2003). However, another group has reported that PKCδ activates PLD1 as effectively as PKCα (Hodgkin *et al.*, 1999). These conflicting reports indicate that there may be potential PKC isoform-dependent mechanisms involved in the regulation of PLD.

Regulation of PLD by small G proteins ARF and Rho

PLD regulation by small G proteins was originally discovered when a cytosolic factor of approximately 16 kDa could reconstitute GTPγS evoked PLD activity in permeabilised (cytosol-depleted) and isolated membranes from HL-60 cells (Anthes *et al.*, 1991; Geny and Cockcroft, 1992; Geny *et al.*, 1993). The cytosolic factor was purified and identified as ADP-ribosylation factor (ARF) (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). ARFs are small molecular weight G proteins that were originally characterised as cytosolic cofactors involved in the ADP-ribosylation of the Gαs heterotrimeric G protein subunit by cholera toxin (Kahn and Gilman, 1984). They have subsequently been implicated in the coatomer-mediated formation and fusion of Golgi vesicles (Serafini *et al.*, 1991; Donaldson *et al.*, 1992a; Palmer *et al.*, 1993) and in the binding of the clathrin adapter protein AP-1 to Golgi membranes (Stamnes and Rothman, 1993; Traub *et al.*, 1993; Zhu *et al.*, 1998). The ARF-dependent PLD activity present within the Golgi apparatus is thought to be important for vesicle trafficking, which is potentially mediated by the product of PLD activity -

phosphatidic acid (Ktistakis *et al.*, 1995; Ktistakis *et al.*, 1996; Chen *et al.*, 1997). Phosphatidic acid may influence the physical characteristics of the lipid bilayer to facilitate a change in the curvature of the membrane and, subsequently, facilitate efficient vesicle budding (Siddhanta and Shields, 1998; Huttner and Schmidt, 2000). PLD1 activity on internal lysosomal membranes has been shown to increase with the assembly of the AP-2 clathrin proteins (Arneson *et al.*, 1999) and as a consequence of ARF-mediated coatomer formation (Jones *et al.*, 1999). The activation of PLD by ARF is markedly increased with fatty acid modification of ARF, with myristoylated ARF being able to activate PLD much more effectively than unmodified ARF (Massenburg *et al.*, 1994; Brown *et al.*, 1995). The site of ARF interaction on PLD currently remains unidentified.

The involvement of the small G protein Rho in the activation of PLD in neutrophils was discovered by Bowman and colleagues (Bowman *et al.*, 1993). After some contention as to whether Rho could indeed activate PLD in some cell types (Ohguchi *et al.*, 1995; Martin *et al.*, 1996; Ohguchi *et al.*, 1996), it has generally been accepted that the main members of the Rho family RhoA, Rac1 and cdc42 can all activate PLD in a similar manner (Hammond *et al.*, 1995; Hammond *et al.*, 1997) and synergistically with ARF (Kuribara *et al.*, 1995; Siddiqi *et al.*, 1995; Hammond *et al.*, 1997). The fatty acid modification of RhoA can greatly increase potency, with geranylgeranylated RhoA being able to stimulate PLD much more effectively than unmodified RhoA (Hammond *et al.*, 1997). It is now known that in the presence of PIP_2 as a cofactor, ARF, Rho and PKC can interact with and activate PLD to a different extent in different cell types (Provost *et al.*, 1996; Hammond *et al.*, 1997). In 1999, a yeast two-hybrid assay revealed that RhoA could interact with the carboxy-terminal domain of human PLD1 (residues 712-1074) (Yamazaki *et al.*, 1999). Point mutagenesis studies of this region indicated that the mutation of residue 870 from isoleucine (I) to an arginine (R) conferred insensitivity to regulation by Rho family GTPases, but otherwise retained a normal expression and activation profile (Du *et al.*, 2000).

Rho family proteins are implicated in the regulation of the actin cytoskeleton and they have been shown to control stress fibre formation and focal adhesions in different cell types, facilitating cell migration (Ridley and Hall, 1992; Ridley *et al.*, 1992; Xu *et al.*, 2003; Burridge and Wennerberg, 2004). In addition, phosphatidic acid has been implicated in the formation of stress fibres (Ha and Exton, 1993a; Cross *et al.*, 1996; Wakelam *et al.*, 1997; Kam and Exton, 2001) and even PLD itself has been shown to interact with the actin cytoskeleton (Lee *et al.*, 2001; Kusner *et al.*, 2002). RhoA has also been shown to be activated indirectly by the heterotrimeric G proteins of the G12/13 family, via the activation of the GTPase activating protein (GAP), p115RhoGEF (guanine nucleotide exchange factor) (Kurose, 2003). Interestingly, activated RhoB has been demonstrated to inhibit the transport of internalised epidermal growth factor receptor to lysosomes (Gampel *et al.*, 1999) implicating a potential role for Rho family members in the recycling of membrane-associated receptors.

ARF and Rho mediated, G protein-coupled receptor stimulation of PLD

The GPCR stimulated activation of PLD through heterotrimeric G proteins has been well characterised (Cockcroft, 1984; Gelas *et al.*, 1989; Martinson *et al.*, 1989). In 1995, Rumenapp and colleagues demonstrated that PLD activation by the M_3 muscarinic receptor in HL-60 cells was ARF-dependent, as brefeldin A (an inhibitor of the ARF-guanine nucleotide exchange factors known as BIG1/2) could inhibit the response (Donaldson *et al.*, 1992b; Rumenapp *et al.*, 1995; Morinaga *et al.*, 1999). Additionally a role for Rho in the GPCR-mediated stimulation of PLD was implicated when PLD activity could be attenuated by the addition of the specific Rho inhibitors C3 exoenzyme from *Clostridium botulinum* or toxin-B from *Clostridium difficile* (Kuribara *et al.*, 1995; Schmidt *et al.*, 1996). In 1998, the Mitchell group showed that ARFs and Rho could associate with GPCRs to activate PLD *via* a mechanism independent of heterotrimeric Gq protein activation (Mitchell *et al.*, 1998). Furthermore, the signalling of the receptor to PLD *via* ARF and Rho was dependent on the

conserved region at the end of the seventh transmembrane spanning helix that contained the conserved motif NPxxY; receptors found to couple to PLD *via* an ARF/Rho independent mechanism contained the motif DPxxY. The mutation of the aspartate (D) to an asparagine (N), conferred BFA (for ARF) or C3 exoenzyme (for Rho) sensitivity onto BFA/C3-insensitive receptor types (such as the gonadotrophin releasing hormone receptor), and the reciprocal mutation conferred insensitivity onto BFA/C3-sensitive receptors (such as the 5-HT_{2A} receptor), implicating the region as a molecular switch for ARF dependent PLD activation (Mitchell *et al.*, 1998).

The domain structure of PLD

The cloning of the mammalian phosphatidylcholine specific PLDs, PLD1 and PLD2 led to investigations into the domain structure of the isoforms and to comparisons with phospholipase C to determine any regions of homology. It transpired that human PLD1 did not contain specific domain regions in common with PLC family members, such as recognisable SH2 or SH3 domains and that the PIP_2 interacting domain was distinct from that of PLCδ (Hammond *et al.*, 1995). The sequences of other PLD family members, such as the phosphatidylinositol-glycan specific PLD isolated from bovine sera (Scallon *et al.*, 1991), indicated that there were conserved domains within the PLD family group. One of these homologous domains is an invariant charged region with residues HxKxxxxD, known as the HKD motif, present at residues 455-490 and 892-926 in the human PLD1 isoform, which is thought to be important for catalytic activity (Hammond *et al.*, 1995). Subsequent studies using mutagenesis of the lysine (K) to an arginine (R) at position 898 have borne out this hypothesis and the mutant human PLD1 K898R has been shown to be catalytically inactive (Sung *et al.*, 1997). The same mutation at the equivalent residue in the PLD2 isoform, K758R, elicits the same loss of catalytic activity (Sung *et al.*, 1997). It is thought that a loop region within the enzyme allows PLD to fold, the amino- and carboxy-terminals are brought into closer proximity and the two HKD motifs can associate together to form a

catalytic centre (Figure 1.5) (Xie *et al.*, 2000; Leiros *et al.*, 2004). The two HKD motifs, along with two other areas of high homology within the PLD family, the IYIENQFF motifs, make up the conserved regions I, II, III and IV (Figure 1.3) (Morris *et al.*, 1996; Frohman *et al.*, 1999).

Mammalian PLD1 and PLD2 both have Phox homology domains, which are conserved regions found in many proteins, that facilitate protein-protein interactions (Frohman *et al.*, 1999). PLD1 has a Phox homology (PX) domain between residues 81-212 and PLD2 has a PX domain between residues 65-195 (Hammond *et al.*, 1995; Lopez *et al.*, 1998). The presence of the PX domains is critical for PLD activity (Frohman *et al.*, 1999; Sung *et al.*, 1999) and it is regions of the PX domain that may interact with the different PKC isoforms (Zhang *et al.*, 1999b). In addition, the PX domains may be necessary for the modulation of the phospholipid-interacting pleckstrin homology domain (Sugars *et al.*, 2002; Ktistakis *et al.*, 2003).

The ability of PLD to localise to cellular membranes is influenced by fatty acid modification of a PIP_2-interacting pleckstrin homology (PH) domain, present in both mammalian PLD isozymes (Sugars *et al.*, 1999; Sung *et al.*, 1999; Xie *et al.*, 2001). Pleckstrin homology domains are conserved peptide regions within many different types of proteins, which allow recruitment of the protein to phosphoinositides of cellular membranes (Kavran *et al.*, 1998). The PH domain of PLD1 is between residues 219-328 and within PLD2 it is between residues 203-311; palmitoylation occurs on two adjacent cysteine residues in the PH domain of both mammalian PLD isozymes (PLD1 240-241 and PLD2 223-224) to facilitate membrane association (Hammond *et al.*, 1995; Lopez *et al.*, 1998; Sugars *et al.*, 1999; Sung *et al.*, 1999; Xie *et al.*, 2001). Furthermore, PIP_2 has additionally been shown to be necessary as a cofactor for PLD activity (Whatmore *et al.*, 1996) and, although the PH homology domain is required for PLD localisation to membranes, the actual site for PIP_2 that is involved in functional activation has been contentious. It was originally proposed that the site of PIP_2 activation of PLD was the PH domain

Figure 1.5

The crystal structure of a bacterial phospholipase D.

The solved crystal structure for a bacterial phospholipase D from *Streptomyces* at 1.4 Å resolution (Berman *et al.*, 2000; Leiros *et al.*, 2004). The peptide chain is rainbow colour coded, from the blue N-terminus to the red C-terminus. The catalytic centre is formed by the enzyme folding at the loop domain to coordinate the HKD motifs together.

(Hodgkin *et al.*, 2000). However, experiments performed more recently would suggest that although the PH domain facilitates PLD localisation with PIP_2-containing lipid membranes, the site of PIP_2 activation of PLD is present in an arginine and lysine rich sequence in the region 691-712 on PLD1 and 554-575 on PLD2 (Du *et al.*, 2003). This region, in addition to the PH and PX regions, is also necessary for efficient PLD1 translocation to the plasma membrane upon cellular stimulation (Du *et al.*, 2003).

PLD localisation

Phospholipase D activity has been reported in many mammalian cell types (Colley *et al.*, 1997a; Morgan *et al.*, 1997; Brown *et al.*, 1998; Exton, 1999; Hodgkin *et al.*, 1999; Czarny *et al.*, 2000; Vitale *et al.*, 2001). However the subcellular localisation of PLD isozyme activity within cells has remained unclear. Currently it is generally agreed that PLD2 mainly localises to the plasma membrane in most cell types (Colley *et al.*, 1997b; Czarny *et al.*, 2000). However the distribution of PLD1 is more variable depending on the cell type. Most PLD1 activity in resting cells appears to be associated with intracellular perinuclear compartments, including the Golgi apparatus, the endoplasmic reticulum (ER), endosomes and lysosomes (Ktistakis *et al.*, 1995; Chen *et al.*, 1997; Colley *et al.*, 1997a; Morgan *et al.*, 1997; Hodgkin *et al.*, 2000; Freyberg *et al.*, 2001). However some groups have also reported PLD1 activity associated with the plasma membrane and the extent to which this is observed may be dependent on the cell type (Hodgkin *et al.*, 1999; Kim *et al.*, 1999a; Lucocq *et al.*, 2001; Vitale *et al.*, 2001).

It is apparent that PLD1 is more mobile than PLD2, and studies demonstrate that upon cellular stimulation, PLD1 translocates from the intracellular compartment membranes to the plasma membrane (Morgan *et al.*, 1997; Brown *et al.*, 1998; Du *et al.*, 2003). When present at the plasma membrane, the PLD activity of either isozyme has been reported to be primarily localised within caveolin-enriched or cytoskeletal domains (Czarny *et al.*, 1999; Hodgkin *et al.*, 1999; Kim *et al.*, 1999a; Czarny *et al.*, 2000; Xu *et al.*, 2000; Han *et al.*,

2002). PLD is dependent on the phospholipid PIP_2 as a cofactor for activation and so must associate with suitable PIP_2-enriched membranes for activity (Hodgkin *et al.*, 2000). The differences in the distribution of the PLD isozymes within different cell types indicates that the host cell may be an important factor in determining how PLD may behave within the model system chosen.

The 14-3-3 proteins

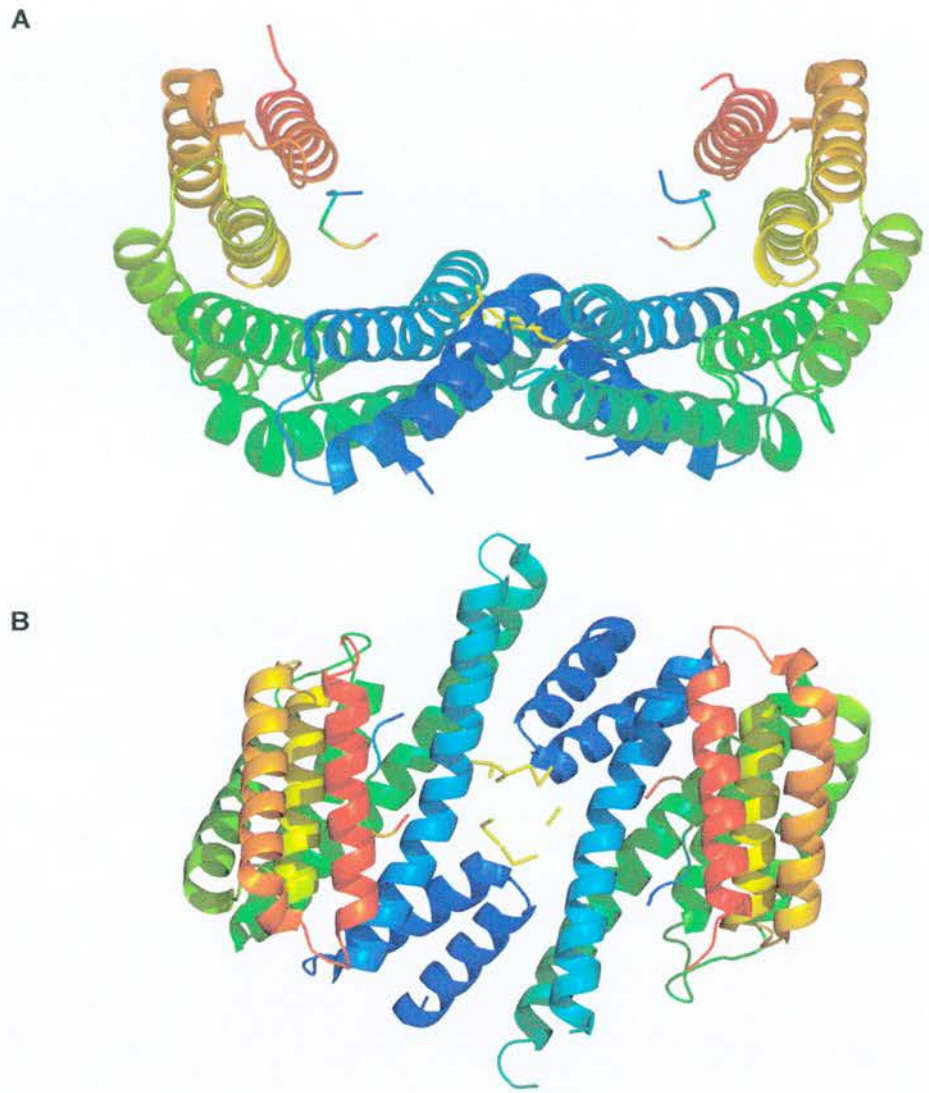
14-3-3 proteins are acidic small molecular weight proteins of approximately 30 kDa that are expressed in all eukaryotic organisms. Initially named due to the unique migration pattern on DEAE cellulose gel electrophoresis (Moore *et al.*, 1968), 14-3-3 proteins have become extensively studied due to their important roles in a great diversity of cellular function. Seven conserved 14-3-3 isoforms are expressed in mammalian cells, β, γ, ε, τ/θ, η, ζ and σ, and are named due to their elution pattern in high performance liquid chromatography (Ichimura *et al.*, 1988; Toker *et al.*, 1990; Toker *et al.*, 1992). Isoforms of 14-3-3 were characterised by a number of different groups working on a number of different mammalian species, however their highly conserved nature and homology allowed them to be correlated successfully (Ichimura *et al.*, 1988; Watanabe *et al.*, 1991; Aitken *et al.*, 1992b; Ichimura-Ohshima *et al.*, 1992; Toker *et al.*, 1992; Leffers *et al.*, 1993; Watanabe *et al.*, 1993; Patel *et al.*, 1994; Roseboom *et al.*, 1994).

Apart from the 14-3-3τ/θ (tau or theta) isoform, which is expressed predominantly in T-lymphocytes (Jones *et al.*, 1995b) and the 14-3-3σ (sigma) isoform (also known as stratifin), which is expressed almost exclusively in epithelial cells (Leffers *et al.*, 1993), the remaining mammalian isoforms are expressed ubiquitously in the brain and peripheral tissues. Up to 1% of the total brain protein level is 14-3-3, suggesting that 14-3-3 proteins are highly expressed and therefore provide a number of important functions in neural cells (Aitken *et al.*, 1992a). 14-3-3 proteins exist *in vivo* as homo- and heterodimers, which are extremely stable and do not readily exchange unless they are denatured and renatured (Jones *et al.*, 1995a). This aspect of 14-3-3 dimerisation has important implications for expressing dominant negative forms of the proteins, as the specificity of dimer formation may influence the functional activity of 14-3-3. The crystal structures of 14-3-3τ (Xiao *et al.*, 1995) and 14-3-3ζ (Liu *et al.*, 1995) were determined in 1995 and indicated that 14-3-3 monomers were made up of nine antiparallel α-helixes and were coordinated together as a dimer to

form a large amphipathic, negatively charged groove. The proximal α-helix of one monomer (residues 5-21) contacts the proximal α-helix of the partner monomer (residues 58-89) to form the base of the groove, the rest of the dimer forming the two opposing faces (Figure 1.6) (Liu *et al.*, 1995; Xiao *et al.*, 1995). Phosphorylation of the serine residue at position 58 by protein kinase B (also known as Akt) has been proposed to negatively regulate the assembly of the 14-3-3 dimer and maintain a monomeric structure (Woodcock *et al.*, 2003). However this site is masked in the stable dimer form (Xiao *et al.*, 1995), so any regulatory phosphorylation *in vivo* would probably need to take place before dimeric 14-3-3 could associate.

The amino acid composition of the groove is highly conserved amongst all isoforms of 14-3-3 (Aitken, 1996). Mutagenesis studies indicated that the residues lysine-49, arginine-56, arginine-127 and a tyrosine-128 (as numbered in 14-3-3ζ) are thought to be important for the interaction with binding partners (and specifically with the coordination of the phosphoserine motif - see below) (Yaffe *et al.*, 1997; Zhang *et al.*, 1997). However, the amino-terminal domains (residues 9-16) and residues on the outer surface of the dimer are variable (Aitken *et al.*, 1992b; Martin *et al.*, 1993; Aitken *et al.*, 1995c). This variability may confer distinct functional roles to different dimer combinations and some target protein interactions. Indeed, studies of the specificity of dimer combinations has revealed that whilst 14-3-3γ generally only forms homodimers (with a small percentage of heterodimers formed with 14-3-3ε), the 14-3-3ε isoform usually only forms heterodimers (with 14-3-3β, 14-3-3η, 14-3-3ζ and 14-3-3γ) (Jones *et al.*, 1995a; Chaudhri *et al.*, 2003). It has been proposed that the dimeric nature of the 14-3-3 proteins may facilitate different cellular mechanisms, with homodimers acting as chaperone proteins and heterodimers acting as functional adapter proteins, coordinating two targets (each binding to a different isoform of the heterodimer) to interact together (Jones *et al.*, 1995a; Aitken *et al.*, 2002). The potential dimeric isoform specificity arising from many 14-3-3 interactions remains to be addressed.

Figure 1.6



The crystal structure of a 14-3-3 dimer.

The solved crystal structure at 2.0 Å resolution of a 14-3-3ζ homodimer complexed with two RSxpSxP phosphopeptides (Rittinger *et al.*, 1999; Berman *et al.*, 2000). The peptide chains are rainbow colour coded on each monomer (the N-terminus is blue, progressing to the red C-terminus). The large amphipathic groove, forming the main channel of the dimer, is bounded on either side by the C-terminal domains of each monomer and is shown from the side (A) and from above (B).

14-3-3 interactions with c-Raf

The serine-threonine kinase c-Raf is a downstream effector of the membrane bound small G protein Ras. Following activation of tyrosine kinase receptors by growth factors, c-Raf is recruited to the plasma membrane where it is activated by Ras in the presence of v-Src and where it subsequently activates the mitogen activated protein kinase (MAPK) (also known as the extracellular signal-regulated kinase (ERK)) pathway by phosphorylating MAPK-kinase (Dent *et al.*, 1992; Kyriakis *et al.*, 1992; Avruch *et al.*, 1994). In 1994 it was discovered that 14-3-3 was a potential binding partner for c-Raf and could be involved in the activation of c-Raf *in vivo*. It appeared to bind readily to c-Raf, and could associate with both inactive c-Raf in cytosol and with activated c-Raf at the plasma membrane (Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994). The conserved regions CR1 and CR2 of c-Raf are found in the amino-terminal domain, which is thought to fold back onto the carboxy-terminal region of the enzyme to negatively regulate the catalytically active CR3 region found in the carboxy-terminal domain (Stanton *et al.*, 1989; Heidecker *et al.*, 1990). The inactive conformation of c-Raf has a high affinity for 14-3-3 dimers rather than monomers *in vivo* (Tzivion *et al.*, 1998). The 14-3-3 interaction sites on c-Raf were determined by tryptic digests of the phosphorylated c-Raf protein and affinity binding studies to peptide lengths of the protein. 14-3-3 was found to interact with c-Raf within the conserved regions CR2 and CR3, where 14-3-3 specifically recognises the consensus sequence RSxpSxP (where pS is phosphoserine) centred on phosphorylated serine residues 259 (RSTpSTP) and 621 (RSApSEP) (Morrison *et al.*, 1993; Muslin *et al.*, 1996). The kinase that phosphorylates c-Raf is quite likely to be protein kinase A (Dhillon *et al.*, 2002; Dumaz and Marais, 2003). The exact nature of the function of 14-3-3 and c-Raf association is still uncertain - some groups have proposed that 14-3-3 mediates the translocation of inactive c-Raf to the plasma membrane and dissociates upon c-Raf activation by Ras (helping recycle c-Raf back to the cytosolic compartment) (Roy *et al.*, 1998), whilst others claim that the continual presence of 14-3-3 is necessary as a cofactor for the maintenance of

c-Raf activity (Thorson *et al.*, 1998; Tzivion *et al.*, 1998). In addition, 14-3-3 may remain bound to active c-Raf at the distal serine site (residue 621) but dissociate from c-Raf at the proximal serine site (residue 259), to coordinate c-Raf into a catalytically active conformation by allowing the protein to unfold (and expose the catalytic CR3 region) (Rommel *et al.*, 1996; Yip-Schneider *et al.*, 2000). Competitive inhibition of the c-Raf binding site by short peptides has been shown to interfere with lipid-dependent activation of the MAPK pathway in *Xenopus* oocytes (Radziwill *et al.*, 1996). However, mutation of the serine-259 to alanine in c-Raf resulted in undiminished MAPK activation, with only a loss of cellular proliferation (Dhillon *et al.*, 2003). Furthermore, a number of reports have indicated that the recruitment of c-Raf to the plasma membrane may be dependent upon the action of phosphatidic acid as a cofactor (Rizzo *et al.*, 1999; Rizzo *et al.*, 2000). PA has been shown to directly bind to c-Raf (between residues 389-423) with high affinity, and inhibition of (PLD-mediated) PA production by pre-treatment with 1% primary alcohol, interrupted 14-3-3 facilitated translocation of c-Raf to the plasma membrane *in vivo* (Ghosh *et al.*, 1996). In addition, mutations of the putative PA binding domain of c-Raf have been shown to cause morphological defects in embryo development, supporting the importance of PA association with c-Raf for full activation of the MAPK pathway (Ghosh *et al.*, 2003).

Protein kinase C can activate c-Raf and the MAPK pathway (see below) in a manner independent of Ras activation (Siegel *et al.*, 1990; Sozeri *et al.*, 1992; Kolch *et al.*, 1993; Ueda *et al.*, 1996; van Dijk *et al.*, 1997). This association is proposed to involve 14-3-3 in a similar way to the coordination of Ras activation of c-Raf, with 14-3-3 behaving as a functional linker to coordinate the two proteins together (Van Der Hoeven *et al.*, 2000b).

14-3-3 interaction with PKC

A family of 29 kDa proteins that potently inhibited protein kinase C (PKC) activity in sheep brain, known as the protein kinase C inhibitor proteins (KCIP), were characterised in 1990 and were shown to be identical in sequence to members of the 14-3-3 family of proteins (Toker *et al.*, 1990; Toker *et al.*, 1992). The characterisation of 14-3-3 as an endogenous PKC inhibitor became complicated by reports that 14-3-3 could activate, rather than inhibit PKC, *in vitro* (Isobe *et al.*, 1992; Tanji *et al.*, 1994). It remains unclear exactly how 14-3-3 can regulate PKC, with many reports finding that 14-3-3 acts as either an inhibitor or activator of PKC (Robinson *et al.*, 1994; Meller *et al.*, 1996; Hausser *et al.*, 1999; Van Der Hoeven *et al.*, 2000a; Gannon-Murakami and Murakami, 2002), although the multiplicity of isoforms for both 14-3-3 and PKC raises the possibility of a broad range of functional interactions. Although the precise mechanism of PKC regulation is unclear, it is apparent that 14-3-3 may associate directly with conventional, atypical and novel PKC isoforms *in vivo* (Meller *et al.*, 1996; Hausser *et al.*, 1999; Van Der Hoeven *et al.*, 2000b; Gannon-Murakami and Murakami, 2002). In T-cells, 14-3-3τ was shown to interact with the novel PKCθ isozyme, and overexpression of 14-3-3τ negatively regulated the PKC mediated activation of the interleukin-2, but not the interleukin-4 promoter (Meller *et al.*, 1996). In PC12 cells, 14-3-3ζ could associate with PKCδ and PKCζ in undifferentiated cells and additionally with PKCε and PKCα in differentiating cells, suggesting the existence of distinct isoform specificity in the association within the cell cycle and differentiation mechanisms (Gannon-Murakami and Murakami, 2002).

Significant matches to the 14-3-3 binding sequence RSxpSxP are present in PKCα, PKCβ and PKCγ, as the motif RRLSVE (residues 238-243) and an additional matching motif RSPSSP is found in PKCγ (686-691) (Muslin *et al.*, 1996). In PKCδ, the interaction motif is thought to be RSDSAS (301-306) and in PKCε, RSKSAP (343-348) (Acs *et al.*, 1995; Aitken *et al.*, 1995a). In PKCι, the recognition sequence may be RSLSVK (474-479)

(Selbie *et al.*, 1993) and in PKCζ the site was demonstrated to be RHDMSYMP (182-189) (Van Der Hoeven *et al.*, 2000a), although this is more similar to a mode-2 phosphoserine interaction (see below). There are also potential interaction sites in PKCη, and PKCθ (Meller *et al.*, 1996; Gannon-Murakami and Murakami, 2002). In addition, the conventional PKC homologue in the organism *Dictyostelium discoideum*, the myosin II heavy chain-specific protein kinase C (MHC-PKC), binds the endogenous Dd 14-3-3 and mammalian 14-3-3ζ within the C1 regulatory domain of MHC-PKC to inhibit kinase activity, and this interaction was independent of phosphorylation on a phosphoserine motif (Matto-Yelin *et al.*, 1997).

The 14-3-3 binding motifs

Recent evidence suggests the phosphoserine-containing 14-3-3 binding motif, typified in c-Raf, may not be the only site for interaction. Most of the 14-3-3 interacting proteins contain one of two phosphoserine based consensus motifs; the mode-1 RSxpSxP or mode-2 RxY/FxpSxP (where pS is phosphoserine) (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The proline residue, although in the cis- conformation in one mode and trans- in the other, is thought to provide a sharp change in chain direction, which allows the consensus target region to exit the binding groove of the 14-3-3 dimer (Obsil *et al.*, 2001; Aitken *et al.*, 2002). Mutagenesis has shown that the residues important in coordinating the binding of 14-3-3 to the phosphoserine consensus sequence are lysine-49 and arginine-56, with contributions from other residues such as arginine-127, tyrosine-128, valine-176 and conserved leucine residues 216, 220 and 227 (numbered as in 14-3-3ζ) (Zhang *et al.*, 1997; Wang *et al.*, 1998). Usually the consensus sequence contains serine residues, however there is no structural reason why threonine residues would not provide a similar interaction domain and indeed many 14-3-3 interacting proteins have phosphorylated threonine residues, such as the serotonin N-acetyltransferase (RRHpTLP) (Aitken *et al.*, 2002). The residue immediately preceding the phosphorylated serine or threonine is often a basic

residue (Aitken *et al.*, 2002). In addition some binding partners contain an alternative, unphosphorylated motif based on the Exoenzyme S recognition sequence WLDLE (Petosa *et al.*, 1998; Masters *et al.*, 1999) or DALDL (Hallberg, 2002). The binding of these motifs is thought to be coordinated by similar interactions within the amphipathic groove of the 14-3-3 dimer, as a peptide based upon the WLDLE sequence (R18 peptide) was able to inhibit c-Raf association and has also been crystallised with 14-3-3 (Liu *et al.*, 1995; Petosa *et al.*, 1998).

Regulation of 14-3-3 interactions and function by phosphorylation

In addition to 14-3-3 recognising a specific phosphorylated motif in many of the 14-3-3 binding partners, the 14-3-3 proteins themselves are regulated by phosphorylation. The 14-3-3α and 14-3-3δ isoforms were shown to be identical to the 14-3-3β and 14-3-3ζ isoforms except they were phosphorylated on a serine residue at position 185 (Martin *et al.*, 1993; Aitken *et al.*, 1995b). This phosphorylation event was able to alter the regulatory properties of 14-3-3 in the interaction with PKC (increasing the inhibition of PKC by nearly two-fold) (Aitken *et al.*, 1995b). Another important regulatory modification of 14-3-3 is the phosphorylation of 14-3-3ζ and 14-3-3τ isoforms by casein kinase 1α (Dubois *et al.*, 1997). This phosphorylation occurs on a threonine residue at position 233 (serine in 14-3-3τ) and has been shown to act to reduce 14-3-3 affinity for binding c-Raf, as only the non-phosphorylated form of 14-3-3ζ interacts with the amino-terminal domain of c-Raf (Rommel *et al.*, 1996; Dubois *et al.*, 1997). The phosphorylation of 14-3-3ζ could therefore potentially yield an isoform specific level of regulation, which may be particularly effective roles of 14-3-3 isoforms in coordinating intracellular signalling pathways.

14-3-3 and apoptosis

The involvement of 14-3-3 in the apoptotic pathway was demonstrated when 14-3-3 was found to bind to the pro-apoptotic protein Bad (Zha *et al.*, 1996). The binding of 14-3-3 to a phosphorylated serine residue at position 136 sequestered Bad into the cytosol and prevented it binding mitochondrial membrane associated Bcl-2 or Bcl-X_L, thereby triggering the apoptotic pathway (Zha *et al.*, 1996; Masters and Fu, 2001; Masters *et al.*, 2001). This binding could be competed off with a 16-mer peptide corresponding to the phosphorylated RSxpSxP motif - [phosphoserine^{259}]-Raf 252-265 (pS-Raf259 phosphopeptide) (Yang *et al.*, 2001). Subsequently it has been shown that 14-3-3ε is an endogenous target for caspase-3, which cleaves 14-3-3ε at Asp-238 to impair the regulatory 14-3-3/Bad interaction, driving the cell into apoptosis by facilitating Bad interaction with Bcl-X_L (Won *et al.*, 2003). Furthermore, 14-3-3 has been shown to bind to (and negatively regulate by sequestering into the cytosol) other pro-apoptotic proteins such as the forkhead transcription factor FKHRL1 (Brunet *et al.*, 1999), Ask1 (Zhang *et al.*, 1999a) and Nur77 (Masuyama *et al.*, 2001), which are transcription factors involved in the activation of various genes involved in apoptosis (Brunet *et al.*, 1999). This implicates 14-3-3 as an important regulatory partner in apoptotic mechanisms.

14-3-3 and receptors

In 1999, Prezeau and co-workers reported that the 14-3-3ζ isoform could interact with the third intracellular loop of the α_2 adrenergic G protein-coupled receptor (Prezeau *et al.*, 1999). Additionally it has been reported that 14-3-3ζ and 14-3-3η isoforms can interact with the carboxy-terminal tail of the metabotropic $GABA_B$ GPCR (Couve *et al.*, 2001). 14-3-3 proteins have also been shown to interact with phospholipid membranes in an isoform-dependent manner (Roth *et al.*, 1994; Jones *et al.*, 1995b). Among other 14-3-3 binding partners are a number of transmembrane spanning proteins, including the tyrosine kinase-linked insulin-like growth factor receptor, ion channels, CLIC4 and the plant plasma

membrane H^+-ATPase (Marra *et al.*, 1994; Craparo *et al.*, 1997; Furlanetto *et al.*, 1997; Suginta *et al.*, 2001; Yuan *et al.*, 2003). Furthermore, 14-3-3 has been shown to facilitate trafficking of the insulin receptor substrate (IRS-1), an important downstream effector involved in the signalling mechanism of the insulin receptor, from the membrane to cytosolic compartments (Craparo *et al.*, 1997; Kosaki *et al.*, 1998; Xiang *et al.*, 2002). This translocation was shown to be dependent on phosphatidylinositol 3-kinase activity and was proposed to be a mechanism of desensitisation of the insulin-dependent activation of IRS-1 (Xiang *et al.*, 2002). Phosphatidylinositol 3-kinase (PI3K) is an enzyme that phosphorylates phosphatidylinositols on the D3 hydroxyl position to produce phosphatidylinositol-3-phosphates. For example, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), to convert it to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PI3K has been shown to bind to 14-3-3 directly at the p110 catalytic subunit and the activity of the enzyme is attenuated in the presence of high levels of 14-3-3 (Bonnefoy-Berard *et al.*, 1995). The potential effects of 14-3-3 in PI3K-mediated signalling mechanisms (which has implications for cell cycle regulation and proliferation) indicates that 14-3-3 may have a functional role in other phospholipid-dependent signalling processes.

In addition, 14-3-3 has been shown to interact with the GTPase activating proteins (GAPS) for the heterotrimeric G proteins, the regulator of G protein signalling (RGS) proteins RGS3 and RGS7 (Benzing *et al.*, 2000; Niu *et al.*, 2002). 14-3-3 proteins have been shown to bind to a phosphorylated serine in the Gα-interacting domain of the RGS protein to inhibit the GTPase activating properties of the protein (Benzing *et al.*, 2000). In doing so, 14-3-3 proteins may act on the transmission of the heterotrimeric G protein signal and help to modulate the signalling of the system following activation, or they may act as scavengers, keeping a pool of the RGS proteins in a negatively regulated state (Niu *et al.*, 2002).

The hypothesis of this study

The nature of 14-3-3 interaction with the third loop of the α_2-adrenergic G protein-coupled receptor and the tail of the metabotropic $GABA_B$ receptor indicates that 14-3-3 may have some physiological role that it can accomplish whilst in association with certain GPCRs. This association may be of functional significance in regulating the signalling of these receptors. In this study, the possibility that 14-3-3 can associate in a similar way with the M_3 muscarinic receptor and the potential location of interaction is investigated.

Phospholipase D contains the sequence Y**RSLSYP**FLL at amino acid residues 711-720 and this motif is identical to the consensus 14-3-3 interaction site, based on the c-Raf sequence RSxpSxP. The implication of a possible direct interaction of 14-3-3 with PLD as well as the possibility that 14-3-3 may interact with the M_3 receptor (as it does with other GPCRs) suggests that 14-3-3 may have an important role in regulating PLD (and perhaps other) signalling mechanisms by this receptor.

The project is therefore designed to investigate the possibility of physical and functional interactions between each of the key proteins of interest – the M_3 receptor, 14-3-3 and phospholipase D.

Chapter 2:

Materials and Methods

Materials and Methods

Plasmids used in this study

Plasmids encoding N-terminally haemagluttinin tagged human phospholipase D1b (HA-PLD1) and mouse phospholipase D2 (HA-PLD2) in pCGN vector were kindly gifted by Dr M. Frohman. The plasmid encoding GST fusion protein of the third intracellular loop of the human M_3 muscarinic receptor (residues Gly308-Leu497) in pGEX 3X was kindly gifted by Dr S. Lanier. Plasmids encoding GST fusion protein of the C-terminal tail domain of the human M_3 muscarinic receptor (residues Asn450-Leu590) in pGEX 2T and the N-terminally signal FLAG tagged human M_3 muscarinic receptor (sFM_3) in pcDNA 3.1 were created and gifted by Dr E. Lutz in collaboration. Plasmids encoding GST fusion proteins of the 14-3-3ζ and 14-3-3η isoforms in pGEX 2T-1 and those encoding the C-terminally myc-tagged wild type 14-3-3ζ, T233D 14-3-3ζ mutant and wild type 14-3-3ε in pcDNA 3.1 were created by Dr T. Dubois. The original wild type 14-3-3η clone was a gift from Dr H. Leffers and was subcloned into pZeo 3.1 by Dr T. Dubois to make the N-terminally myc-tagged 14-3-3η. The deletion mutant Δ^-26 14-3-3ε in pcDNA 3.1 was created by Dr D. Jones.

Cell lines

COS 7 green monkey kidney fibroblast cells were used throughout this study. The culture media used was Dulbecco's Modified Eagle's Media (DMEM) (with 4500 mg/l glucose and l-glutamine but without pyruvate) supplemented with 10% normal calf serum (NCS) and the antibiotics penicillin and streptomycin at 50 units/ml. All tissue culture media and normal calf serum in this study was obtained from Gibco (Invitrogen Life Technologies, Paisley, UK).

Cell culture

Cell lines were grown and maintained in a humidified atmosphere of 5% CO_2 (v/v) at 37 °C at all times unless otherwise indicated. Culture medium was refreshed every 3-4 days, all flasks, 6/12 well plates for culturing and plastics were obtained from Greiner Bio-One (Gloucestershire, UK).

The cells were harvested by a brief incubation with 10 ml/175 cm^2 Hank's Buffered Saline Solution (HBSS) containing 10% (v/v) 10x trypsin-EDTA (Gibco), which was applied to the cell layer, agitated and aspirated after 20 seconds. After a further 10 minutes, the cells were washed off the flask surface with the appropriate medium and resuspended. The cells were then reseeded into flasks at a ratio of 1:3 or onto 6 or 12 well plates for microscopic or assay purposes.

For storage purposes, 0.5 ml volumes of cells at 10^6 cells/ml in medium or foetal calf serum containing 7-10% (v/v) dimethylsulfoxide (DMSO) were aliquoted into cryosafe tubes and stored in an insulted polystyrene box at –70°C overnight. The tubes were then transferred to the liquid nitrogen storage facility. To recover the cells from the cryostore, the tubes were allowed to thaw at room temperature and then warmed in a 37°C water bath for two minutes. The cells were resuspended in 8 ml medium, transferred to a 25 cm^2 flask and incubated overnight. The medium was then refreshed to remove any trace of DMSO and the cells cultured as normal.

Preparation of mammalian cDNA

I. Transformation of competent cells

A 200 µl aliquot of either JM109 or TOP10 competent strains of *Escherichia coli* was added to 1 µg of plasmid cDNA, carefully mixed and incubated on ice for 30 minutes. The cells were subjected to heat shock at 42 °C for 45 seconds in a water bath and placed back on ice immediately. Following a further incubation of 5 minutes, 400 µl of sterile Luria Bertani

(LB) medium (10 g/l bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl; pH 7.5) was added to the cell mixture and incubated with rotation at 37 °C for 1 hour. 100 µl of this suspension was then spread onto LB-agar (LB medium containing 1% (w/v) bactoagar) plates, supplemented with 50 µg/ml ampicillin for plasmid selection and incubated overnight at 37 °C. A single discrete colony was picked and inoculated into 3 ml of sterile LB medium containing 100 µg/ml ampicillin (LB-amp) and incubated with agitation for four hours at 37 °C. This culture was then seeded into 100 ml LB-amp in a sterile 250 ml conical flask (to allow sufficient aeration) and incubated overnight at 200 rpm in an orbital shaking incubator at 37 °C.

II. Plasmid purification

Plasmid cDNA was purified using QIAfilter Plasmid Maxi Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4 °C and all traces of medium were removed. The pellet was resuspended in 10 ml ice cold Resuspension Buffer P1 (50 mM Tris-HCl pH 8.0, 10mM EDTA) containing 100 µg/ml RNase A solution until homogeneous. Following resuspension, 10 ml of Lysis Buffer P2 (200 mM NaOH, 1% (w/v) SDS) was added and mixed gently by inversion. This was incubated at room temperature for 5 minutes precisely. Subsequently 10 ml of chilled Neutralisation Buffer P3 (3.0 M potassium acetate; pH 5.5) was added and mixed thoroughly to prevent further lysis. The mixture of supernatant and fluffy white precipitate (containing genomic DNA, cell debris and SDS) was then poured immediately into a previously prepared QIAfilter Maxi cartridge and allowed to incubate at room temperature for 10 minutes. During the cartridge incubation, a QIAGEN-tip 500 column was equilibrated with 10 ml Equilibration Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). Following incubation, the bacterial lysate was filtered into the QIAGEN-tip 500 and the cleared supernatant

allowed to flow through. The tip was then washed with 2 x 30 ml Wash Buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol) and the DNA eluted into an ethanol washed polypropylene tube using 15 ml Elution Buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol). The DNA was precipitated by adding 10.5 ml isopropanol at room temperature, mixed and centrifuged at 15 000 x g for 30 minutes at 4°C (Sorvall SS-34 rotor). The pellet was carefully washed with 5 ml of sterile 70% (v/v) ethanol at room temperature and centrifuged at 15 000 x g for 10 minutes. The pellet was washed a further time and the pellet subsequently air dried for 10 minutes before being resuspended and dissolved in approximately 200 µl sterile TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA; pH 7.6).

Transient transfection of cDNA into mammalian cells

COS 7 cells were trypsinised as described earlier and seeded into 75 cm^2 or 175 cm^2 flasks or onto 6 or 12 well plates at approximately 50% confluency for transfection the following day. Fugene-6 transfection reagent (Roche Diagnostics, East Sussex, UK) or Genejuice (Novagen, Merck Biosciences, Nottingham, UK) was added to DMEM (with 4500 mg/l glucose, with l-glutamine, without pyruvate) according to the manufacturers instructions and incubated for 5 minutes. cDNAs encoding proteins of choice or empty vector pcDNA3 (as a control) were added to the Fugene-6/DMEM or Genejuice/DMEM according to the manufacturers instructions and swirled gently to mix, then incubated for a further 15 minutes. The transfection mixture was added to the cell layer, swirled gently and the cells incubated at 37 °C for 48 hours. Cells were made quiescent by replacing culture media with serum free media.

Preparation of cell extracts overexpressing specified proteins

COS 7 cells used for preparing cellular extracts were seeded into 175 cm^2 flasks and transfected with relevant cDNAs as previously described. Cells were made quiescent approximately four hours before lysis. The cells were washed once in HBSS and solubilised in 2 ml/175 cm^2 flask Extraction buffer (PBS pH 7.5 containing 20% (w/v) glycerol, 1.0% (w/v) CHAPS, 1.0% (w/v) sodium deoxycholate, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM AEBSF) on ice for 1 hour with occasional agitation. The cellular lysate and debris was scraped into two 1.5 ml tubes on ice and centrifuged at 12 000 x g for 20 minutes at 4 °C. Each supernatant was then transferred to a clean tube and was reserved on ice for immediate use in GST fusion studies and the remaining pellet was lysed to check for any detergent insoluble fractions with x2 Laemmli buffer (50 mM Tris, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) mercaptoethanol) containing 8 M urea for efficient running of membrane associated proteins (Sung *et al.*, 1999).

Glutathione S-transferase (GST) fusion studies

I. Transformation of competent cells

The procedure for transforming competent bacterial JM109 or BL-21 RIL *E. coli* strains with plasmids encoding GST-fusion constructs was carried out as described previously for the preparation of mammalian cDNA; however transformed bacterial colonies were selected for by plating cells onto 2x YT (16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl; pH 7.0) supplemented with 1.5% (w/v) bacto-agar and 100 μg/ml ampicillin.

II. Bacterial growth and expression of fusion proteins

A single selected bacterial colony from the 2x YT selection plate was used to inoculate 20 ml of 2x YT medium containing 2% (w/v) glucose (2x YTG) and 100 μg/ml ampicillin in a sterile 250 ml conical flask and was incubated overnight at 200 rpm in an orbital shaking

incubator at 37 °C. This overnight culture was then seeded at a ratio of 1:100 (4 ml) into 400 ml 2x YTG with 100 μg/ml ampicillin in a sterile 2 l flask and incubated at 37 °C until the optical density at a wavelength of 600 nm (A_{600}) of the culture reached between 0.4 and 0.6 - indicative of the culture reaching exponential growth. The *tac* promoter (*Ptac*) of the pGEX GST plasmid requires isopropyl-β-D-thiogalactopyranoside (IPTG) in order to induce protein production from the open reading frame (ORF) that encodes the GST gene and inserted DNA sequences. Bacterial expression of the specific GST fusion protein was therefore induced by adding IPTG to yield a final concentration of 0.5 mM per culture and incubated for a further 3 hours. The culture was transferred to 2 x 300 ml centrifuge bottles (Nalgene, Hereford, UK) and the cells pelleted by centrifugation at 7700 x g for 10 minutes at 4 °C (Sorvall GSA rotor). The pellet was washed with 20 ml PBS, centrifuged again, drained and stored overnight at –40 °C.

III. GST fusion protein purification

The bacterial pellet was allowed to thaw at room temperature and then gently resuspended until homogeneous in 10 ml BugBuster reagent (Novagen, Merck Biosciences, Nottingham, UK). The resulting lysate was centrifuged at 16 000 x g for 20 minutes at 4 °C. The clear supernatant was reserved and the pellet discarded. Each bacterial supernatant was diluted appropriately to allow for efficient comparison of GST-construct binding. To this was added 400 μl of a glutathione sepharose 4B suspension (1:1 glutathione sepharose 4B:PBS) (Amersham Biosciences) and the mixture was incubated for 20 minutes at room temperature with rolling. The beads were pelleted by centrifugation at 3000 x g for 2 minutes at 4 °C and the supernatant removed. The beads were washed with 2 ml PBS and again pelleted, this wash process was repeated twice more. Samples of enriched COS 7 cell extracts were incubated with the GST fusion protein-linked glutathione beads in the absence or presence of 100 μM [$Pser^{259}$]-Raf 252-265 (Raf259 phosphopeptide) where indicated overnight at 4

°C with rolling. The beads were then pelleted by centrifugation at 3000 x g for 2 minutes and the supernatants removed. The beads were washed with 1 ml cold extraction buffer and again pelleted, this wash process was repeated a further three times. Finally the supernatants were removed and the associated proteins were solubilised from the matrix by adding 80 µl x2 Laemmli buffer containing 8 M urea and stored at –20 °C for electrophoresis and immunoblot analysis.

[^{3}H]N-Methyl-QNB binding to immunoprecipitates

Cells used in co-immunoprecipitation studies were seeded into 175 cm^2 flasks and transfected with relevant cDNAs as previously described. Cells were made quiescent 16 hours before lysis and if required, agonist stimulation was carried out for 20 minutes unless otherwise described. The cells were washed once in HBSS and solubilised in 2 ml/175 cm^2 flask immunoprecipitation (IP) buffer (PBS pH 7.5 containing 20% (v/v) glycerol, 1.0% (w/v) CHAPS, 1.0% (w/v) sodium deoxycholate, 2 µg/ml aprotinin, 4 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM AEBSF) on ice for 1 hour with occasional agitation. The cellular lysates and debris was scraped into two 1.5 ml tubes on ice and centrifuged at 12 000 x g for 20 minutes at 4 °C. Each supernatant was then transferred to clean 1.5 ml tubes and 20 µl of a Protein G-sepharose (PrG) suspension (1:1 PrG beads:IP buffer) was added to 'preclear' the lysate and remove any non-specific antibody interaction, this incubation was carried out for 1 hour with rolling at 4 °C. The pellets were reserved and resuspended in 1 ml QNB binding buffer (50 mM $NaPO_4$, 2 mM $MgCl_2$; pH 7.4, 2 µg/ml aprotinin, 1% (w/v) BSA) before being centrifuged at 12 000 x g for 30 minutes at 4 °C. The pellets were resuspended in 1 ml QNB binding buffer and stored at –40 °C. The precleared cellular supernatants were incubated overnight with primary antibody or species specific non-immune IgG as a negative control with 40 µl PrG suspension, in the absence or presence of 100 µM [Pser^{259}]-Raf 252-265 (Raf259 phosphopeptide) where indicated, rolling at 4 °C. Following

incubation, the supernatants were reserved, to 500 µl was added 100 µl of 0.1% (w/v) bovine gamma globulin in PBS and 600 µl 30% (w/v) polyethylene glycol (PEG; 8000 wt) in PBS, the samples were mixed and centrifuged for 15 minutes at 12 000 x g at 4 °C. The supernatant was discarded and the PEG pellet was resuspended in 1.2 ml QNB binding buffer. The immunoprecipitate beads were washed twice with 1 ml IP buffer and resuspended in 1.2 ml QNB binding buffer. Samples of 200 µl of each condition were aliquoted into clean tubes to yield appropriate triplicates of total binding compared to duplicates of non-specific binding (a total of 5 x 200 µl per condition). A final concentration of 2.38 nM [^{3}H]N-methyl-quinuclindinyl benzilate ([^{3}H]NMe-QNB; 3.1 TBq/mmol) (Kd = 0.58 ± 0.04 nM in COS 7 cells) (Mitchell *et al.*, 2003) was added to all the tubes in a volume of 25 µl. An additional 25 µl of buffer was added in the tubes where total binding was being assessed, whereas in the tubes determining non-specific binding, 25 µl of 1 µM final concentration N-methyl atropine was added. Ligand binding was carried out by incubation at room temperature for 4 hours. Binding was quenched by addition of 250 µl of cold QNB binding buffer to each sample, followed by 100 µl of 0.1% (w/v) bovine gamma globulin in PBS and 600 µl 30% (w/v) polyethylene glycol (PEG; 8000 wt) in PBS. The samples were centrifuged at 12 000 x g for 10 minutes at 4 °C and aspirated carefully. The tips of the tubes containing the PEG pellets were cut from each sample into 6 ml Pony 'hang-in' vials (Perkin Elmer, Bucks, UK) and 4 ml of scintillation fluid (Perkin Elmer) was added to each. Vials were capped and shaken briefly, incubated overnight at room temperature and then counted on a Beckman LS 5801 series scintillation counter (Beckman, Bucks, UK) using a 4 minute/sample, [^{3}H] decays per minute (DPM) program.

Co-immunoprecipitation

Cells used in co-immunoprecipitation studies were seeded into 175 cm^2 flasks and transfected with relevant cDNAs as previously described. Cells were made quiescent 16

hours before lysis and if required, agonist stimulation was carried out for 10 minutes unless otherwise described. The cells were washed once in HBSS and solubilised in 2 ml/175 cm^2 flask immunoprecipitation (IP) buffer (PBS pH 7.5 containing 20% (v/v) glycerol, 1.0% (w/v) CHAPS, 1.0% (w/v) sodium deoxycholate, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM AEBSF) on ice for 1 hour with occasional agitation. The cellular lysates and debris was scraped into two 1.5 ml tubes on ice and centrifuged at 12 000 x g for 20 minutes at 4 °C. Each supernatant was then transferred to clean 1.5 ml tubes and 20 μl of a Protein G-sepharose (PrG) suspension (1:1 PrG beads:IP buffer) was added to 'preclear' the lysate and remove any non-specific antibody interaction, this incubation was carried out for 1 hour with rolling at 4 °C. The pellet was reserved and lysed with x2 Laemmli buffer (50 mM Tris, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) mercaptoethanol) containing 8 M urea to check for any detergent insoluble fractions. During the 'preclear' incubation, the antibody-Protein G conjugate was prepared by adding primary antibody (or an equivalent concentration of species specific non-immune IgG as a negative control) to 40 μl of the PrG:IP suspension in another clean 1.5 ml tube and this was incubated for 1 hour at 4 °C with rolling. Following the 'preclear' step, the mixture was centrifuged at 12 000 x g for 30 seconds at 4 °C to pellet the Protein G beads and 1 ml of the supernatant transferred to the antibody-PrG conjugated beads for incubation overnight at 4 °C with rolling. The remainder of the precleared supernatant was reserved and an equal volume of x2 Laemmli buffer containing 8 M urea was added to quantify the transfection efficiency and check the levels of protein present in the cells. Following overnight incubation, the solution was centrifuged at 12 000 x g for 30 seconds to pellet the Protein G beads and the supernatant was removed. The Protein G was resuspended in 1 ml IP buffer and then pelleted by centrifugation. This wash process was repeated twice more. The Protein G pellet was finally resuspended in 40 μl x2 Laemmli buffer containing 8 M urea and stored at –40 °C for electrophoresis and immunoblot analysis.

SDS-PAGE and immunoblotting (Western blot)

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting was performed using two systems, depending on the sample type and volume. The procedure used for immunoblotting was common to both methods.

I. PhastSystem (Amersham Biosciences)

The manufacturers instructions were followed using 12.5% pre-cast homogeneous polyacrylamide gels (PhastGel Homogeneous 12.5, Amersham Biosciences, Bucks, UK). The gel was placed, excluding air bubbles, film side down onto 80 µl of UHP H_2O on the pre-cooled (16 °C) gel bed of a PhastSystem separation and control unit (Amersham Biosciences). The buffer strip assembly was placed upon the gel and PhastGel SDS buffer strips (Amersham Biosciences) were placed into the holder, ensuring an even contact was made with the gel underneath. The electrode assembly was then put into place, ensuring an even contact with the buffer strips. The samples for SDS-PAGE (4 µl/lane) were loaded onto the sample strip holder and this was placed in the apparatus. The separation program was pre-set (at 250 V, 16 °C) and the samples were run for 90 AVh (approximately 35 minutes). During this time, Immobilon-P^{SQ} polyvinyldifluoride (PVDF) transfer membrane (0.2 µM pore size, 20 cm x 20 cm, Millipore, Gloucestershire, UK) was prepared by cutting to 5 cm x 5 cm and marked for orientation. The membrane was immersed in 100% methanol and then washed briefly five times with UHP H_2O on a rotary shaker. The membrane was then incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol; pH 8.3) with three changes until PAGE separation was completed.

Following PAGE, the gel was removed from the bed and the gel separated from the film backing using the clamp and wire apparatus (Amersham Biosciences) at the interface of the stacking and separating gel. The Immobilon-P^{SQ} membrane was placed onto the separating gel, ensuring that the orientation was correct and the plastic film back removed. Filter

papers (3 pieces of 5 cm x 5 cm) were soaked in transfer buffer and placed onto the anode plate of the PhastSystem development unit and the Immobilon-P^{SQ} placed directly onto them (gel uppermost). Another three filter papers were soaked in transfer buffer and placed on top of the gel/Immobilon-P^{SQ}. The cathode block was connected to the assembly and placed carefully on top of the filters. The system was then switched on and transferred for 80 minutes using another automatic program (20 V, 16 °C).

II. Invitrogen NuPAGE Novex gel and XCell *SureLock* Mini-Cell system

Prior to PAGE, samples for separation were prepared by adding 2 µl of 10x Loading buffer (0.04% (w/v) bromophenol blue in glycerol) to 20 µl of sample (in x2 Laemmli buffer). This loading sample was then vortexed to mix, centrifuged for 2 minutes at 14 000 rpm and heated to 70 °C for 5 minutes. A 4-12% Novex Bis-Tris pre-cast NuPAGE gradient gel (Invitrogen, Paisley, UK) was placed into an XCell *SureLock* Mini-Cell (Invitrogen) according to the manufacturers instructions. The inner buffer chamber was filled with 200 ml of 1x MES (50 mM MES, 50 mM Tris base, 1 mM EDTA, 0.1% (w/v) SDS; pH 7.3) or 1x MOPS (50 mM MOPS, 50mM Tris base, 1mM EDTA, 0.1% (w/v) SDS; pH 7.7) SDS running buffer containing 500 µl NuPAGE antioxidant, the outer buffer chamber was filled with 600 ml of 1x MES or 1x MOPS SDS running buffer. A sample volume of 20 µl was loaded into each well. The mini-cell was connected to a power supply that provided a constant potential of 200 V and was run for 45 minutes (MES buffer) or 55 minutes (MOPS buffer). Meanwhile, Immobilon-P^{SQ} polyvinyldifluoride (PVDF) transfer membrane (0.2 µM pore size, 20 cm x 20 cm, Millipore) was prepared by cutting to 9 cm x 7.5 cm and marked for orientation. The membrane was immersed in 100% methanol and then washed briefly three times with UHP H_2O on a rotary shaker. The membrane was then incubated in 1x NuPAGE transfer buffer (25 mM bicine, 25 mM bis-tris, 1mM EDTA, 10% (v/v) methanol; pH 7.2) with three changes until PAGE separation was completed. Following

separation, the gel was removed from the plastic cassette and the 'foot' was removed. The membrane was placed on top of the gel (excluding any air bubbles) and two pieces of filter paper pre-soaked in 1x NuPAGE transfer buffer were used to sandwich the gel/membrane on each side. Three pre-soaked blotting pads were then placed in the cathode core of the blot module, the paper/gel/membrane sandwich was placed onto the pads (membrane uppermost) and three pre-soaked blotting pads were placed on top of the arrangement. The anode plate was put into place and the module was put into the mini-cell carefully, ensuring the assembly remained airtight. The inner chamber of the blot module was filled with remaining 1x NuPAGE transfer buffer and the outer chamber of the mini-cell was filled with UHP H_2O. The mini-cell was connected to the power supply and run at a constant 30 V for 80 minutes.

III. Immunoblotting

The Immobilon-P^{SQ} membrane was removed form the appropriate transfer system, briefly rinsed and given five washes in UHP H_2O to remove any residual transfer buffer. The membrane was then stained for 2 minutes in 0.1% (w/v) coomassie blue-R250 (Amersham Biosciences), 50% (v/v) methanol and subsequently destained in 50% (v/v) methanol, 10% (v/v) acetic acid until dark protein bands were contrasted with a pale background. The membrane was washed in UHP H_2O and placed into a clear polythene sheet for documentary visualisation of protein loading. The membrane was scanned using Adobe Photoshop 7.0 (Adobe Systems, CA, USA) software on an Apple Macintosh. The membrane was cut as appropriate and all stain was removed using several washes of 100% methanol. The membrane was washed again in UHP H_20 (5 x 5 minutes) to remove any residual methanol and blocked overnight in 5% (w/v) non-fat dried milk (Marvel) in PBS at 4 °C. Following blocking, the membrane was washed 5 x 5 minutes in 0.1% (v/v) tween-20 in PBS and incubated with an appropriate primary antibody concentration in primary buffer

(2.0% (w/v) non-fat dried milk, 0.05% (v/v) tween-20 in PBS) for 90 minutes at room temperature. The membrane was washed at least 5 x 5 minutes in 0.1% (v/v) tween-20 in PBS. The membrane was incubated with an appropriate horseradish peroxidase conjugated secondary antibody concentration (raised against the species used for the primary) in secondary buffer (2.0% (w/v) BSA; 0.1% (v/v) tween-20 in PBS) for 50 minutes at room temperature. Following secondary antibody incubation, the membrane was washed at least 5 x 5 minutes in 0.1% (v/v) tween-20 in PBS. The membrane was visualised using enhanced chemiluminescence (ECL) detection methods. LumiGLO reagent and peroxide (Cell Signalling Technology, NEB, Herts UK) were mixed in a 1 in 20 dilution and incubated on the membrane for 1 minute. Excess ECL solution was removed and the membrane sealed in a clear polythene bag. The membrane was exposed to ECL film (Amersham Biosciences) in an X-ray cassette for between 30 seconds and 20 minutes and the film developed using an automatic X-ray developer (Konica-Minolta, Bucks, UK). The film was scanned using Adobe Photoshop 7.0 (Adobe Systems, CA, USA) software on an Apple Macintosh. Any quantitative densitometry values were derived using the ScanAnalysis program (Biosoft, Cambridge, UK).

Immunocytochemistry

I. Slide preparation

COS 7 cells used in immunocytochemistry were first plated onto sterile 2.5 mm^2 glass coverslips in 6 well dishes and transfected with relevant cDNAs as described previously. Cells were made quiescent 16 hours before stimulation. Agonist stimulation was carried out for 10 minutes unless otherwise indicated. Following stimulation, the medium was aspirated and the cells permeabilised and fixed using 1 ml/well of ice cold 1:1 HBSS (w/o Ca^{2+} or Mg^{2+}): 4% (w/v) paraformaldehyde (PFA), 0.3% (v/v) Triton X-100 in PBS. The cells were incubated for 16 hours at 4 °C. Following incubation, the cells were aspirated and the fix

medium was replaced with 1 ml/well 4% PFA, 0.3% Triton X-100 in PBS and incubated for 10 minutes at room temperature. This was aspirated and the cells washed twice with 1 ml/well 0.2% (v/v) fish skin gelatin (FSG) in PBS. Following washes, the cells were incubated with 1 ml/well 2% (w/v) BSA, 0.05% (v/v) tween-20 in PBS for 40-60 minutes at room temperature to block non-specific antibody binding. The cells were then washed twice with 1 ml/well 0.2% FSG in PBS. Primary antibody incubation was carried out by inverting each coverslip onto 200 µl primary antibody buffer (primary antibody in 2% BSA, 0.05% tween-20 in PBS) spotted onto a polythene sheet and then incubated for an hour at room temperature. The cells were returned to the 6 well plates and washed five times with 1 ml/well 0.2% FSG in PBS with agitation. Secondary antibody incubation was then carried out using 1 ml/well secondary antibody buffer - Alexa Fluor conjugated secondary antibodies: green (488 nm) anti-mouse and red (568 nm) anti-rabbit, 1:1000 in 0.2% FSG in PBS (Molecular Probes, Leiden, The Netherlands) - for 30 minutes in the dark with agitation. The cells were washed five times with 1 ml/well 0.2% FSG in PBS in the dark. The coverslips were briefly washed in UHP H_2O and then mounted onto glass microscope slides (BDH, Dorset, UK) using approximately 50 µl movial mounting fluid per coverslip, ensuring no air bubbles formed. The slides were left overnight at room temperature and subsequently stored at 4 °C in the dark before use.

II. Image acquisition

All data were acquired using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) with a PlanApochromat 63x/1.4 Oil DIC objective. Cells were imaged using scanning with a double dichroic excitation beam splitter; emission wavelengths corresponding to 488 nm excitation were collected through a Band Pass 525/550 nm filter, and emission wavelengths corresponding to 568 nm excitation were collected through a Long Pass 590 nm filter. The pinhole was set to 1 Airy Unit and scan

speed to maximum. Images were acquired at Nyquist sampling rates at 8 bit per pixel depth and all scanned frames were sequentially tracked for each channel with a minimum average of four passes to reduce noise.

II. Image analysis

Image data were deconvolved using the Huygens 2 software package (Scientific Volume Imaging, Hilversum, The Netherlands) on SGI Octane II workstations. Each channel was subjected to a maximum of 20 iterative algorithms to reach quality threshold levels for reducing blur and noise. Optical aberration and the point-spread-function were determined in software using a maximum likelihood estimation (MLE) algorithm. The data was subsequently analysed using the Bitplane Imaris suite of image analysis software (Bitplane AG, Zurich, Switzerland). The total number of colocalised voxels above thresholds per channel was measured along with proportional amounts for each channel. The channel correlation coefficient was determined from the whole dataset according to the Pearson method (Bitplane AG).

Assay for Phospholipase C activity: [^{3}H]inositol phosphate production

COS 7 cells used in phospholipase C (PLC) activity assays were first plated into 12 well dishes and transfected with relevant cDNAs as described previously. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) throughout the assay. Prior to the assay, cells were made quiescent in EBSS (Earle's balanced salt solution) with 10mM HEPES and 1.8% (w/v) glucose. The cells were labelled with 37 KBq/well myo-[2-[^{3}H](N)]inositol (37 MBq/ml, 888 GBq/mmol, Perkin-Elmer, Bucks, UK) for 16 hours. Following incubation, the labelling medium was aspirated and the cells washed twice with 1 ml/well EBSS containing 10mM HEPES, 1.8% (w/v) glucose and 0.2% (w/v) BSA (fractionV) and then incubated with 0.5 ml/well of the same medium for approximately 20

minutes. 10 minutes prior to stimulation, 10 µl of 500 mM LiCl was added to yield a final concentration of 10 mM Li^+ per well. The cells were stimulated with agonist and incubated for 30 minutes unless otherwise indicated. The assay was stopped by aspirating the assay medium and adding 1 ml of ice cold 10 mM formic acid per well (Almaula *et al.*, 1996) and incubating overnight at 4°C. Each sample was then carefully applied to a 1 ml bed volume Dowex anion exchange column (1x8 resin; formate form; 200-400 mesh) and a stepwise gradient of ammonium formate was used to elute the [^{3}H]inositol phosphate products as described by Berridge (Berridge *et al.*, 1983).

Each column was washed with:

15 ml of UHP water

5 ml of 50 mM ammonium formate

The [^{3}H]inositol phosphates were eluted and collected into 20 ml Zinsser scintillation vials (Zinsser analytic, Berks, UK) using:

10 ml of 1.0 M ammonium formate/0.1 M formic acid

The columns were then washed with:

5 ml of 2.0 M ammonium formate/0.1 M formic acid

15 ml UHP water

Following collection of the eluted products, 500 µl of each eluate was aliquoted in duplicate into 6 ml Pony 'hang-in' polyethylene vials (Perkin-Elmer) and 4 ml of Emulsifier-safe liquid scintillation cocktail (Perkin-Elmer) was added. Vials were capped and shaken briefly, incubated overnight at room temperature and counted on a Beckman LS 5801 series scintillation counter (Beckman, Bucks, UK) using a 4 minute/sample, [^{3}H] decays per minute (DPM) program.

Assay for Phopholipase D activity: [^{3}H]phosphatidyl butanol production

COS 7 cells used in phospholipase D (PLD) activity assays were first plated into 12 well dishes and transfected with relevant cDNAs as described previously. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) throughout the assay. Prior to the assay, cells were made quiescent in DMEM (w/o sodium pyruvate, with 4500 mg/l glucose, with pyroxidine HCl) and the fatty acid moiety of phosphatidylcholine was labelled with 185 KBq/well [9,10-^{3}H]palmitate (185 MBq/ml, 1 TBq/mmol, Perkin Elmer) for 16 hours. Following incubation, the labelling medium was aspirated and the cells were washed once with 0.75 ml/well MEM (Eagles; with 25 mM HEPES, with Earles salts, w/o l-glutamine) containing 1.0% (w/v) essentially fatty acid free BSA and then incubated with 0.5 ml/well of the same medium. Immediately prior to the assay, Butan-1-ol in MEM was added to each well to give a final concentration of 30 mM butan-1-ol per well. The assay was initiated with agonist and incubated for 30 minutes unless otherwise stated. The assay was terminated by the aspiration of assay medium and addition of 500 µl ice cold methanol per well. Cells were scraped and the extraction of [^{3}H]PtdBut product followed a method of lipid extraction described by Bligh and Dyer (Bligh and Dyer, 1959). Samples were transferred to 2 ml glass vials (Chromacol, Merck/BDH) and 500 µl chloroform and 400 µl H_2O were added to yield a ratio of methanol:chloroform:water of 5:5:4. They were vortexed, capped with rubber seals, and left overnight at 4 °C. The samples were centrifuged for 20 minutes at 1000 rpm to separate the aqueous and organic layers. The upper aqueous layer was discarded and 250 µl of the lower organic layer was transferred to new clean vials. The solvent was evaporated overnight or under vacuum at 30 °C in a centrifugal evaporator (Gyrovap, V A Howe, Oxfordshire, UK) for 30-50 minutes. Each sample was resuspended in 50 µl of a solvent mixture of 19:1 chloroform:methanol and spotted onto a lane of a LK5D 150 Å silica gel thin layer chromatography (TLC) plate (250 µm thick) (Whatman, Kent, UK). The plates were developed using the organic phase of the

running solvent mixture (ethyl acetate, 2,2,4-trimethylpentane, acetic acid, water, in the ratio 110:50:20:100). The region of TLC plate corresponding to the migration of [^{3}H]PtdBut had been identified previously using a [^{14}C]PtdBut standard (McCulloch, 1998). Six segments of 0.5 cm length per lane, from between 4.0-7.0 cm from the plate origin, were scraped into six 6 ml Pony 'hang-in' polyethylene vials (Perkin-Elmer) and 4 ml of Emulsifier-safe liquid scintillation cocktail (Perkin-Elmer) was added. Vials were capped and shaken briefly and counted on a Beckman LS 5801 series scintillation counter (Beckman, Bucks, UK) using a 4 minute/sample, [^{3}H] decays per minute (DPM) program.

Data analysis

All quantitative values from assays were expressed as mean ± standard error of the mean (SEM) acquired from a number (n) of individual experiments. Statistical analysis was carried out using the Wilcoxon matched pairs nonparametric test unless otherwise indicated. The Graphpad Prism program (Graphpad Software, CA, USA) was used to analyse and plot the data.

All Research Collaboratory for Structural Bioinformatics (RCSB) protein databank (pdb) molecular visualisations were performed using the PyMOL Molecular Graphics System software (DeLano Scientific, CA, USA). Quantitative densitometry values were derived using the ScanAnalysis program (Biosoft, Cambridge, UK).

Chapter 3:

The interaction of 14-3-3 with the M_3 muscarinic receptor

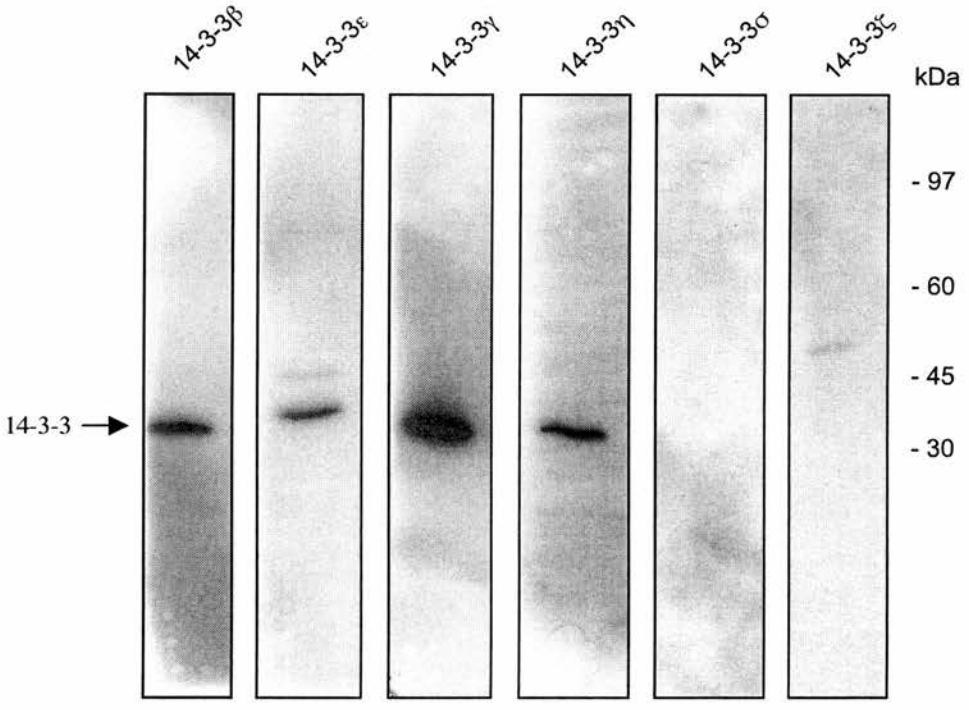
Introduction

Experiments carried out by Prezeau and colleagues in 1999 revealed the unexpected finding that 14-3-3ζ could bind to the third intracellular loop of subtypes A-C of the α_2-adrenergic receptor (Prezeau *et al.*, 1999). Whilst much work had been done on other 14-3-3 binding partners, this was the first indication that 14-3-3 could interact with G protein-coupled receptors and suggested that 14-3-3 may be providing a scaffolding or even functional role by this association. It was subsequently reported that 14-3-3ζ and 14-3-3η isoforms could interact with another GPCR, the metabotropic $GABA_B$ receptor, within the carboxy-terminal tail domain, potentially to regulate the heterodimerisation or functionality of the receptor (Couve *et al.*, 2001). In addition, 14-3-3 has been shown to interact with the regulator of G-protein signalling (RGS) proteins RGS3 and RGS7 (Benzing *et al.*, 2000; Niu *et al.*, 2002). The 14-3-3 protein appears to sequester RGS proteins by binding to a phosphorylated serine and inhibiting the GTPase activating properties of these proteins. These results together suggest that 14-3-3 may provide a direct structural role in GPCR scaffolding by binding to receptors directly, or 14-3-3 may provide a functional role by binding to partners involved in signalling cascades or their associated proteins and such interaction may act to regulate receptor-mediated signalling pathways. To determine whether 14-3-3 proteins could potentially interact with the M_3 muscarinic receptor in a similar way to the findings of other groups with the α_2 adrenergic and $GABA_B$ receptors, the experimental approaches of *in vitro* glutathione S-transferase fusion protein pull-down assays and co-immunoprecipitation studies were used. In addition, the isoform specificity of 14-3-3 involved in any potential interaction was investigated to find out whether 14-3-3 isoforms differed in their properties of association with the M_3 receptor. Any such finding of isoform selectivity would suggest the potential for fine control of 14-3-3:M_3 receptor interactions (and of any further cellular events dependent on these interactions).

The native 14-3-3 isoforms in COS 7 cells

Many of the isoforms of 14-3-3 proteins are expressed abundantly in the peripheral tissues and brain – up to 1% of brain protein is 14-3-3 (Aitken *et al.*, 1992). In order to understand the effects of expressing additional 14-3-3 constructs, it was necessary to determine which 14-3-3 isoforms were endogenously present in the COS 7 cell line. Untransfected serum-deprived COS 7 cells were lysed on ice in cold CHAPS/deoxycholate-containing extraction buffer, proteins were separated by SDS-PAGE and Western blotted. Different endogenous 14-3-3 isoforms were visualised using rabbit polyclonal primary antisera specific to each of the isoforms (Martin *et al.*, 1993) followed by anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Chemicon, Hants, UK) and the results are shown in Figure 3.1. COS 7 cells were found to contain reasonably high levels of endogenous 14-3-3β, 14-3-3ε, 14-3-3γ and 14-3-3η, and much lower levels of 14-3-3ζ and 14-3-3σ isoforms. As it was the 14-3-3ζ isoform that was determined to interact with the α_2 adrenergic receptor (Prezeau *et al.*, 1999) and 14-3-3ζ was additionally implicated in the interaction with the metabotropic $GABA_B$ receptor (Couve *et al.*, 2001), we considered that additional expression of myc-tagged 14-3-3ζ or other 14-3-3 isoforms may be required to optimally observe the influence of these proteins on M_3 receptor function. Therefore myc-tagged 14-3-3 isoforms (N-terminally myc-tagged 14-3-3η and C-terminally myc-tagged 14-3-3ε and 14-3-3ζ) were transiently transfected into COS 7 cells. The fact that these were myc-tagged yielded an extra level of control within the model system, so that isoform specificity could be determined (especially as the normally poorly expressed 14-3-3ζ was a likely candidate) and allowed for greater sensitivity with regard to detection of 14-3-3 using high affinity antibodies for the myc-tag of the specific 14-3-3 isoform transfected.

Figure 3.1



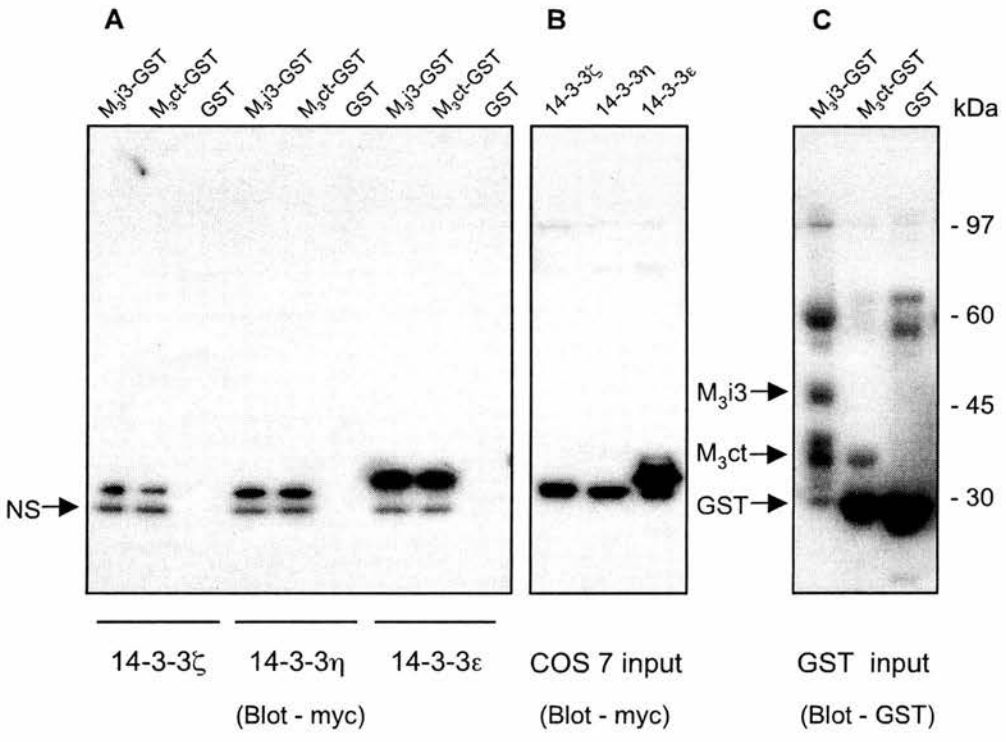
Endogenous 14-3-3 isoforms in COS 7 cells.

Samples of native COS 7 cell extract were separated by SDS-PAGE and immunoblotted with specific rabbit polyclonal primary antibodies (Martin *et. al.*, 1993) to determine native expression of six of the isoforms of 14-3-3 proteins. The β, ε, γ, and η isoforms of 14-3-3 were found to be normally expressed at readily detectable levels in COS 7 cells. COS 7 cells appeared to express very little of the isoforms 14-3-3σ and 14-3-3ζ.

14-3-3 isoforms interact with the M_3 receptor in vitro

To determine whether 14-3-3 proteins could interact with the intracellular domains of the M_3 muscarinic receptor, an *in vitro* binding assay was used. Glutathione S-transferase (GST) fusion proteins of the third intracellular loop of the M_3 receptor (M_3i3), corresponding to residues G308-L497, and the C-terminal domain of the M_3 receptor (M_3ct), corresponding to residues N540-L590, were kindly gifted by Dr S. Lanier and Dr E. Lutz respectively. These GST fusion proteins and GST alone (as a negative control) were immobilised onto glutathione Sepharose 4B and incubated overnight with CHAPS/deoxycholate-solubilised cellular extracts from COS 7 cells that had been transiently overexpressing myc-tagged 14-3-3 isoforms (14-3-3ζ-myc, 14-3-3ε-myc and myc-14-3-3η). Bound proteins were lysed from the beads, separated by SDS-PAGE, and Western blotted. Myc-tagged 14-3-3 isoforms were visualised using rabbit anti-myc polyclonal primary antibody (Upstate Biotech, Milton Keynes, UK) followed by HRP-conjugated anti-rabbit secondary antibody (Chemicon). Input levels of 14-3-3-myc immunoreactivity in extracts were also monitored and both fusion protein and binding protein inputs were carefully balanced as far as possible to ensure comparability between samples. The GST-fusion constructs, visualised using rabbit polyclonal anti-GST (Santa Cruz, Autogen Bioclear Ltd., Wilts, UK), expressed with different efficiencies, however only bands that corresponded to the intact constructs were taken into account when balancing the GST-fusion input levels (Figure 3.2). The three 14-3-3 isoforms specifically associated with both the third intracellular loop and carboxy-terminal tail domains of the M_3 muscarinic receptor and the binding of 14-3-3ζ but not 14-3-3η or 14-3-3ε exhibited interaction site specificity. Although the 14-3-3ε association with the M_3 receptor constructs showed an apparently stronger binding than the other 14-3-3 isoforms, the difference in expression levels of the 14-3-3ε isoform may have been sufficient to account for the higher apparent binding levels. Densitometric analysis of the Western blot bands suggested that in contrast to the other 14-3-3 isoforms, 14-3-3ζ displayed a lower

Figure 3.2



14-3-3 isoforms bind to the M_3 receptor *in vitro*.

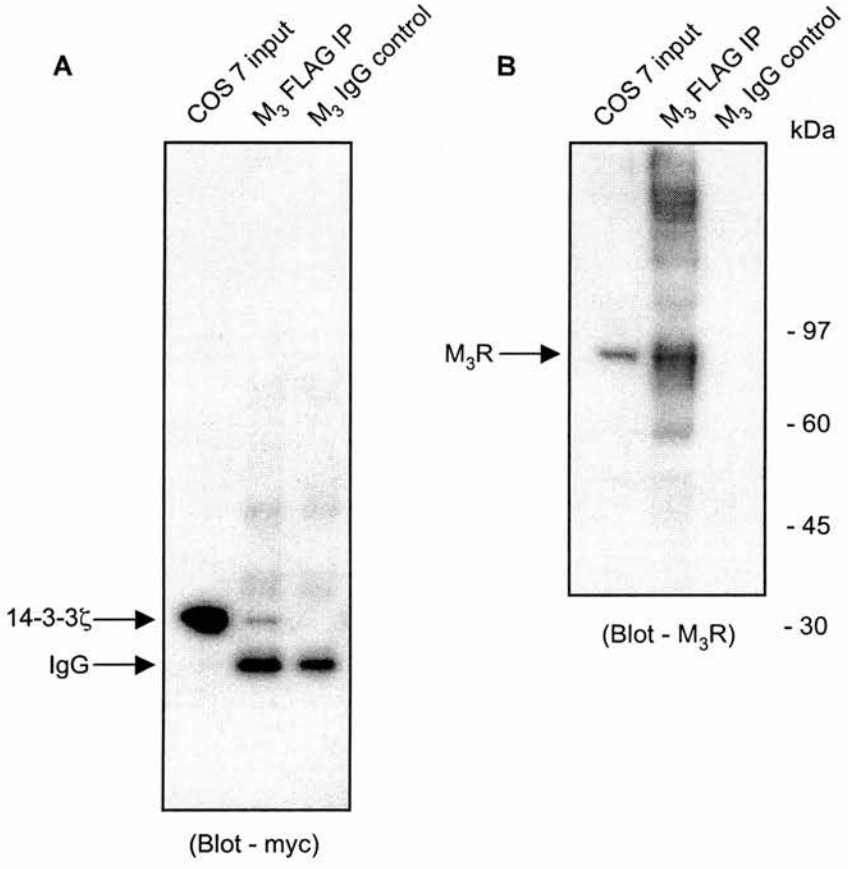
Extracts of COS 7 cells, transiently overexpressing different myc tagged 14-3-3 isoforms were applied to GST-fusion constructs of the M_3 receptor third intracellular loop (M_3i3) and tail (M_3ct) domains. The immunoblot shows the binding of the three isoforms of 14-3-3 to the constructs (A) compared to input levels of protein (B) (both probed for the myc tag) and input levels of GST (blotted for GST) (C). A non-specific band, which was immunoreactive in all preparations, is indicated (NS). The GST reactive bands above 60 kDa are probably due to breakdown products in the bacterial expression system.

affinity for the M_3 carboxy-terminal tail domain compared to the third intracellular loop domain (a reduction in band grey scale density of approximately 30% given balanced inputs of constructs and potential binding protein in each case) and similar findings were made in three independent experiments. This parallels the findings made with subtypes of the α_2-adrenergic receptor (where 14-3-3ζ specifically interacted with the third intracellular loop) (Prezeau *et al.*, 1999). The 14-3-3ε and 14-3-3η isoforms, however, bound to both GST-M_3i3 and GST-M_3ct constructs with a similar affinity, suggesting that any molecular interactions of these isoforms with the receptor *in vivo* may be provided by a contribution of both the loop and tail domains.

14-3-3 isoforms co-immunoprecipitate with the M_3 receptor

COS 7 cells were transiently transfected with signal FLAG tagged M_3 (sFM_3) receptor and myc-tagged 14-3-3ζ (14-3-3ζ-myc) cDNA in a 1:1 ratio and were serum-deprived for 16 hours prior to the experiment. Immunoprecipitates directed against the FLAG tag were prepared by incubating precleared supernatants from CHAPS/deoxycholate-containing cellular extracts with 3 μg/ml mouse monoclonal anti-FLAG antibody clone M2 (Sigma Aldrich, Dorset, UK) or 3 μg/ml non-immune mouse IgG as a control, followed by 40 μl/ml of a 1:1 suspension of Protein-G Sepharose in immunoprecipitation buffer. The beads were washed and co-immunoprecipitates lysed from the matrix using Laemmli buffer, separated by SDS-PAGE, Western blotted and visualised using rabbit anti-myc primary antibody (Upstate Biotech) followed by HRP-conjugated anti-rabbit secondary antibody (Chemicon). The input levels of immunoprecipitated M_3 receptor were visualised with rabbit polyclonal anti-M_3 receptor primary antibody (kindly gifted by Dr A. Tobin), followed by anti-rabbit HRP-conjugated secondary antibody (Chemicon). Figure 3.3 shows the co-immunoprecipitation of the 14-3-3ζ isoform with the M_3 receptor and the levels of immunoprecipitated receptor (and is representative of the findings from two independent

Figure 3.3



14-3-3ζ co-immunoprecipitates with the M_3 receptor.

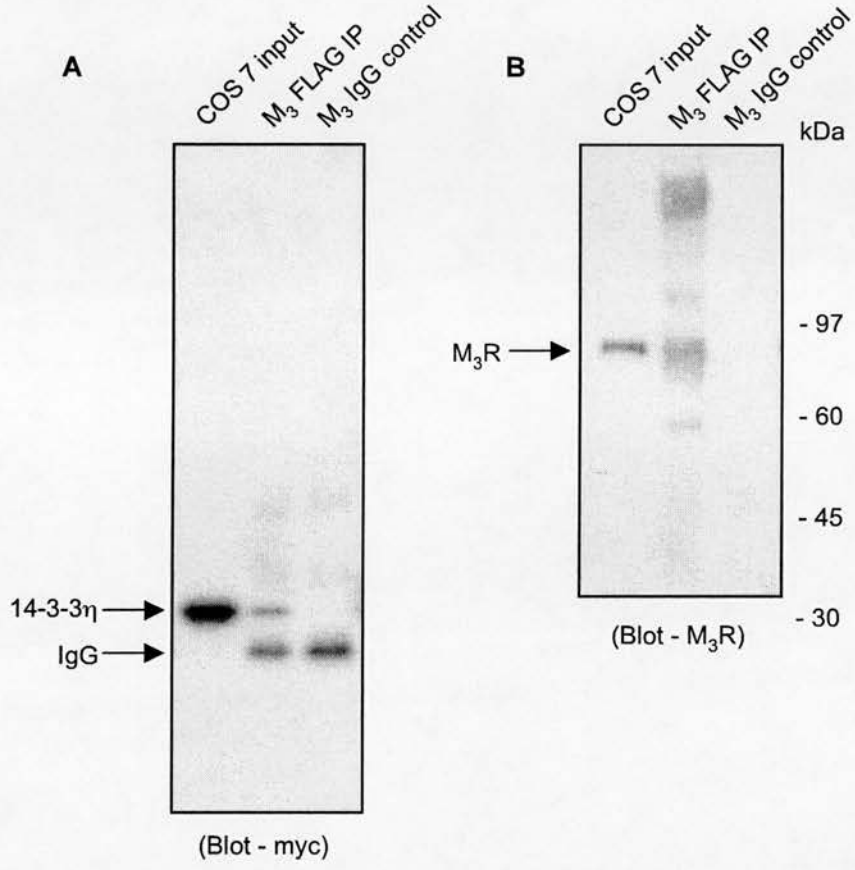
COS 7 cells were transiently transfected with sFM_3 receptor and 14-3-3ζ-myc constructs. Immunoprecipitation was made with anibody directed against the FLAG tag of the receptor and co-immunoprecipitated 14-3-3 levels were visualised by probing Western blots for the myc tag (A). The levels of FLAG immunoprecipitate of the sFM_3 receptor, probed with anti-M_3 antibody (A. Tobin), are shown in (B). A sample of the cellular supernatant was applied as a positive control (COS 7 input) and the non-specific antibody bands resulting from the reagent input are indicated (IgG). The M_3 receptor specifically pulls down the 14-3-3ζ isoform compared to the control.

experiments). This interaction corroborates the finding of the *in vitro* binding assay, where the 14-3-3ζ bound to the third intracellular loop of the M_3 receptor (apparently with a higher affinity than to the tail) and corroborates the findings of Prezeau and colleagues who observed 14-3-3ζ associating with the third loop of the $\alpha_{2A\text{-}C}$-adrenergic receptors. However, in the study from Prezeau and co-workers, the co-immunoprecipitation of 14-3-3ζ with the α_2-adrenergic receptor subtypes could not be demonstrated (Prezeau *et al.*, 1999), indicating that any interaction of 14-3-3 with the M_3 receptor is either more stable, or of a higher affinity, than that with the α_2-adrenergic receptor.

These immunoprecipitation experiments were repeated with 14-3-3η, to determine whether there was any isoform specificity in the association. COS 7 cells were transiently co-transfected with the sFM_3 receptor and myc-14-3-3η cDNAs at a 1:1 ratio and were serum deprived for 16 hours. The M_3 receptor was immunoprecipitated from the precleared CHAPS/deoxycholate cellular lysates with antibody against the FLAG tag of the M_3 receptor (anti-FLAG, clone M2, Sigma) or non-immune mouse IgG (as a control) and the levels of co-immunoprecipitated 14-3-3η were visualised by SDS-PAGE, Western blotting and probing for the myc-tag (Figure 3.4). As with the 14-3-3ζ isoform, the M_3 receptor pulled down 14-3-3η and the interaction appeared to be quite robust. In the studies demonstrating 14-3-3 association with the metabotropic $GABA_B$ receptor, it was the 14-3-3ζ and 14-3-3η isoforms that were shown to associate with the carboxy-terminal tail domain of the receptor (Couve *et al.*, 2001). In that study, other 14-3-3 isoforms were not tested, so it was important to determine whether 14-3-3 and M_3 receptor association was selective with respect to 14-3-3 isoforms, by using a third 14-3-3 isoform.

The 14-3-3ε-myc isoform was transfected together with the sFM_3 receptor into COS 7 cells and the M_3 receptor was immunoprecipitated using the FLAG tag exactly as before. A representative result of the co-immunoprecipitation of 14-3-3ε with the M_3 receptor is shown in Figure 3.5. As was the case with the other isoforms, 14-3-3ε was shown to

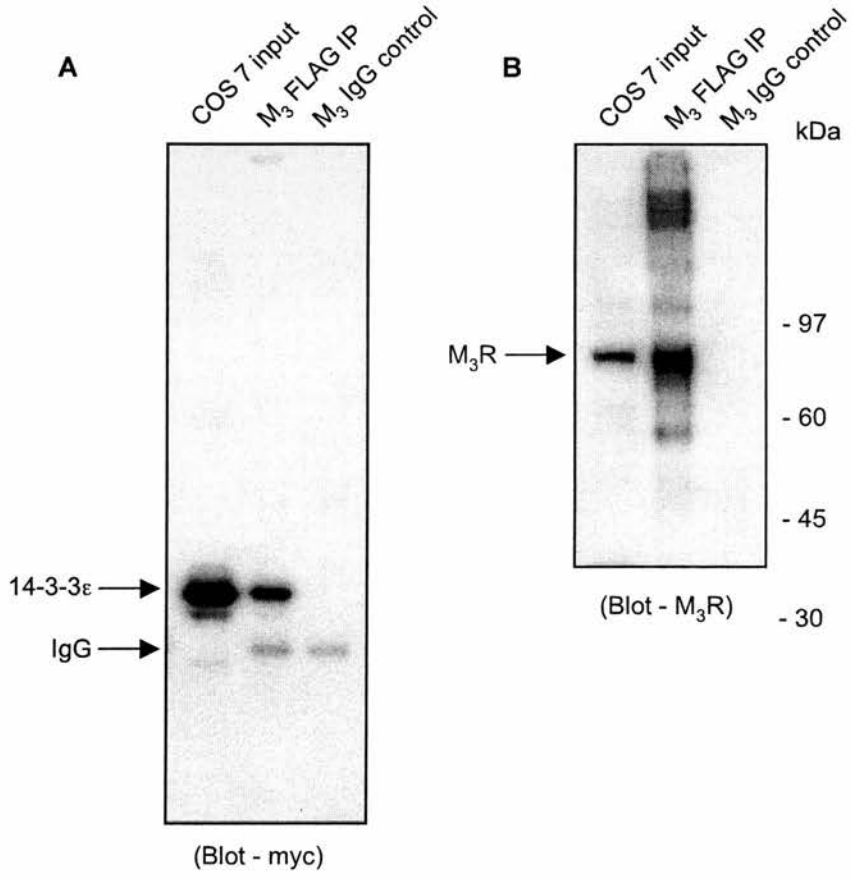
Figure 3.4



14-3-3η co-immunoprecipitates with the M_3 receptor.

COS 7 cells were transiently transfected with sFM_3 receptor and myc-14-3-3η constructs. Immunoprecipitation was made with anibody directed against the FLAG tag of the receptor and co-immunoprecipitated 14-3-3 levels were visualised by probing Western blots for the myc tag (A). The levels of FLAG immunoprecipitate of the sFM_3 receptor, probed with anti-M_3 antibody (A. Tobin), are shown in (B). A sample of the cellular supernatant was applied as a positive control (COS 7 input) and the non-specific antibody bands resulting from the reagent input are indicated (IgG). The M_3 receptor specifically pulls down the 14-3-3η isoform compared to the control.

Figure 3.5



14-3-3ε co-immunoprecipitates with the M_3 receptor.

COS 7 cells were transiently transfected with sFM_3 receptor and 14-3-3ε-myc constructs. Immunoprecipitation was made with anibody directed against the FLAG tag of the receptor and co-immunoprecipitated 14-3-3 levels were visualised by probing Western blots for the myc tag (A). The levels of FLAG immunoprecipitate of the sFM_3 receptor, probed with anti-M_3 antibody (A. Tobin), are shown in (B). A sample of the cellular supernatant was applied as a positive control (COS 7 input) and the non-specific antibody bands resulting from the reagent input are indicated (IgG). The M_3 receptor specifically pulls down the 14-3-3ε isoform compared to the control.

specifically co-immunoprecipitate with the M_3 receptor, in accordance with the *in vitro* GST association described earlier. This suggests, at least for the isoforms tested here, that 14-3-3 interaction with the receptor may not show any marked isoform specificity.

The binding of 14-3-3 to the M_3 receptor may involve the amphipathic groove

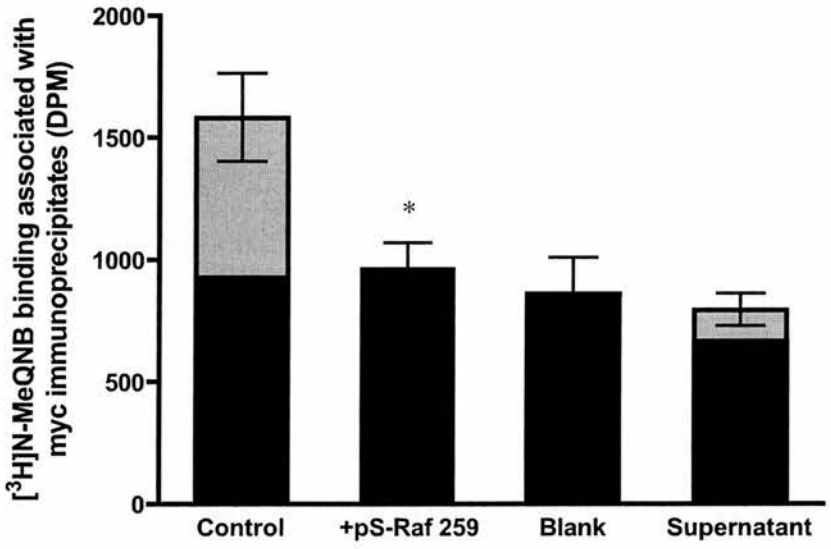
The binding of 14-3-3 to many binding partners depends on the recognition of the c-Raf-like motif RSxpSxP or RxY/FxpSxP (Muslin *et al.*, 1996; Yaffe *et al.*, 1997) whilst other interactions may depend on an unphosphorylated WLDLE (Petosa *et al.*, 1998) or DALDL (Hallberg, 2002) motif. Experiments were carried out to assess whether the binding of 14-3-3 to the M_3 muscarinic receptor could be disrupted by a peptide consisting of the c-Raf phosphoserine motif as a competitive binding partner for the 14-3-3 amphipathic groove. The M_3:14-3-3 interaction was assayed by immunoprecipitating 14-3-3 (for the myc tag) and using specific binding of radiolabelled M_3 receptor antagonist [^{3}H]N-Me-QNB to determine the presence of M_3 receptor co-immunoprecipitating with 14-3-3. COS 7 cells were transiently transfected with signal FLAG-tagged M_3 (sFM_3) receptor and myc-tagged 14-3-3ζ (14-3-3ζ-myc) cDNA in a 1:1 ratio and were serum deprived for 16 hours prior to the experiment. Immunoprecipitates directed against the myc tag were prepared by incubating precleared cellular supernatants with 2 μg/ml mouse monoclonal anti-myc antibody clone 9E10 (Sigma Aldrich, Dorset, UK) or 2 μg/ml non-immune mouse IgG as a control, followed by 40 μl/ml of a 1:1 of Protein-G Sepharose suspension, in the absence or presence of 100 μM [phosphoserine^{259}]-Raf 252-265 (pS-Raf259 phosphopeptide), LSQRQRSTpSTPNVHMV (K_D ~ 120 nM for 14-3-3ζ) (Muslin *et al.*, 1996). The 14-3-3 myc immunoprecipitates, along with the precleared supernatants were assayed for the presence of the M_3 muscarinic receptor binding sites by incubating the immunoprecipitates with 2.4 nM [^{3}H]N-Me-QNB to determine total binding (K_D = 0.58 ± 0.04 nM in COS 7 cells) (Mitchell *et al.*, 2003) or radioligand plus 1 μM N-methyl atropine to determine non-

specific binding (K_D ~ 0.5-2.0 nM) (Maggio *et al.*, 1999; Mitchell *et al.*, 2003), for four hours at room temperature. The immunoprecipitates were centrifuged at 12 000 x g for 10 minutes at 4 °C, washed and assayed for levels of [^{3}H] ligand binding by liquid scintillation counting. The non-specific binding was subtracted from the total binding to yield specific binding (Figure 3.6). Although 14-3-3 has many cellular binding partners (Aitken, 1996), the increased sensitivity of the radiolabelled-antagonist binding assay is able to detect very low levels of associated M_3 receptor. This association was attenuated by the presence of the high affinity 16-mer phosphoserine motif 14-3-3 binding peptide, pS-Raf 259, suggesting that 14-3-3ζ interacts with the M_3 receptor, either directly or indirectly, at a similar site to the phosphoserine peptide, in the amphipathic groove of the 14-3-3 dimer. Further experiments with control peptides (for example scrambled and de-phosphorylated sequences) would enable the specificity of this effect to be fully substantiated and further characterise the mode of interaction.

Membrane localised 14-3-3 is primarily in the plasma membrane fraction

It has been well documented that 14-3-3 is a mainly cytosolic protein (Moore *et al.*, 1968; Boston *et al.*, 1982), yet there is also evidence of 14-3-3 localising with synaptic membranes in neuronal cells (Martin *et al.*, 1994; Jones *et al.*, 1995b) and with the Golgi apparatus (Celis *et al.*, 1988). In addition to this, and because the M_3 receptor is a membrane inserted protein, it was important to determine whether 14-3-3 could associate with cellular membranes and elucidate the localisation of any membrane-associated 14-3-3 in COS 7 cells. To do this, serum-deprived untransfected cellular membranes from COS 7 cells were prepared by Dr Rory Mitchell and fractionated through Percoll gradients (Amersham Biosciences, Bucks, UK) as described in a previous study (Mitchell *et al.*, 2003) to separate endosomal, Golgi and plasma membrane fractions. The membrane fractions 1-10, corresponding to the membranes of different subcellular compartments, were solubilised in Laemmli buffer, separated by SDS-PAGE and Western blotted (Figure 3.7).

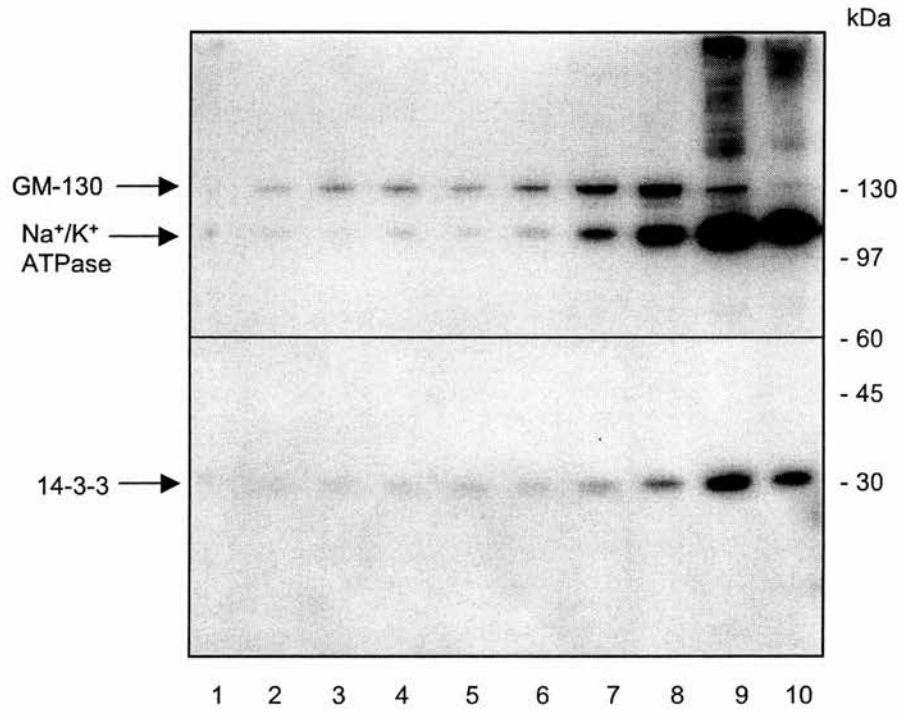
Figure 3.6



The binding of 14-3-3 with the M_3 receptor involves the amphipathic groove of 14-3-3. The immunoprecipitates of 14-3-3ζ-myc under control conditions and in the presence of 100 μM of the 14-3-3 recognition sequence 16-mer peptide pS-Raf 259 (LSQRQRSTpSTPNVHMV) (+pS-Raf 259) were assayed for the presence of M_3 receptor by specific [^{3}H]N-MeQNB binding. The non-specific binding (black bars) was subtracted from the total binding (grey bars) for each condition to yield specific binding. The binding levels of Protein-G Sepharose beads only (Blank) and the binding from the precleared cellular supernatants (Supernatants) are also shown. The presence of the pS-Raf 259 peptide prevents specific binding of the M_3 receptor to 14-3-3ζ immunoprecipitates, indicating that occlusion of the amphipathic groove of 14-3-3 prevents 14-3-3 association with the M_3 receptor (*$p<0.05$, Unpaired student t-test; n=5).

The marker for the Golgi-associated membranes, GM-130 (130 kDa) was visualised using anti-GM-130 mouse monoclonal primary antibody (Transduction Laboratories, BD Biosciences, Cowley, UK) with anti-mouse HRP-conjugated secondary antibody (Chemicon) and was detected most highly in the fractions 7 and 8, with a smaller amount present in fractions 6 and 9. The Na^+/K^+ ATPase α1 subunit (112 kDa), used as the marker for the plasma membrane, was visualised using anti-Na^+/K^+ ATPase α1 mouse monoclonal primary antibody (Upstate Biotech) with anti-mouse HRP-conjugated secondary antibody (Chemicon) and was present most highly in fractions 9 and 10, with smaller amounts in fraction 8. The 14-3-3 levels were visualised using anti-14-3-3 (clone H8, isoform non-specific) mouse monoclonal primary antibody (Santa Cruz) with anti-mouse HRP-conjugated secondary antibody (Chemicon). The 14-3-3 was found in increasing amounts in the later fractions and was present at the highest levels in fractions 9 and 10 - corresponding to the plasma membrane, with a smaller presence in fraction 8 corresponding to the Golgi. These results, which were typical of two independent experiments, indicate that whilst 14-3-3 may predominantly be a cytosolic protein, it has the ability to associate with cellular membranes and specifically the plasma membrane. These findings support previous experimental information from other groups by demonstrating that 14-3-3 has a similar potential localisation to that of the M_3 muscarinic receptor and indicates that under basal conditions, some isoforms of 14-3-3 at least may enter into a complex with the M_3 muscarinic receptor. Indeed, as 14-3-3 has also been shown to localise to the plasma membrane of neuronal cells (Martin *et al.*, 1994; Jones *et al.*, 1995b), the implication that 14-3-3 may play an important functional role in facilitating neurotransmitter activity (by interacting with GPCRs) is quite compelling.

Figure 3.7



Membrane associated 14-3-3 is located primarily at the plasma membrane.

Membrane fractions 1-10 were prepared from resting COS 7 cells using Percoll gradient fractionation as described in Mitchell, *et. al.*, 2003. The Golgi membrane marker GM-130 was visualised with high levels in fractions 7-9, the Na^+/K^+ ATPase was used for identification of the plasma membrane and was present in high levels in fractions 9 and 10. The 14-3-3 was visualised using a pan-isoform specific antibody, and was at highest levels in fractions 9 and 10 - corresponding to the plasma membrane.

Effects of wild-type and mutant 14-3-3ζ expression on the agonist-stimulated signalling properties of the M_3 receptor

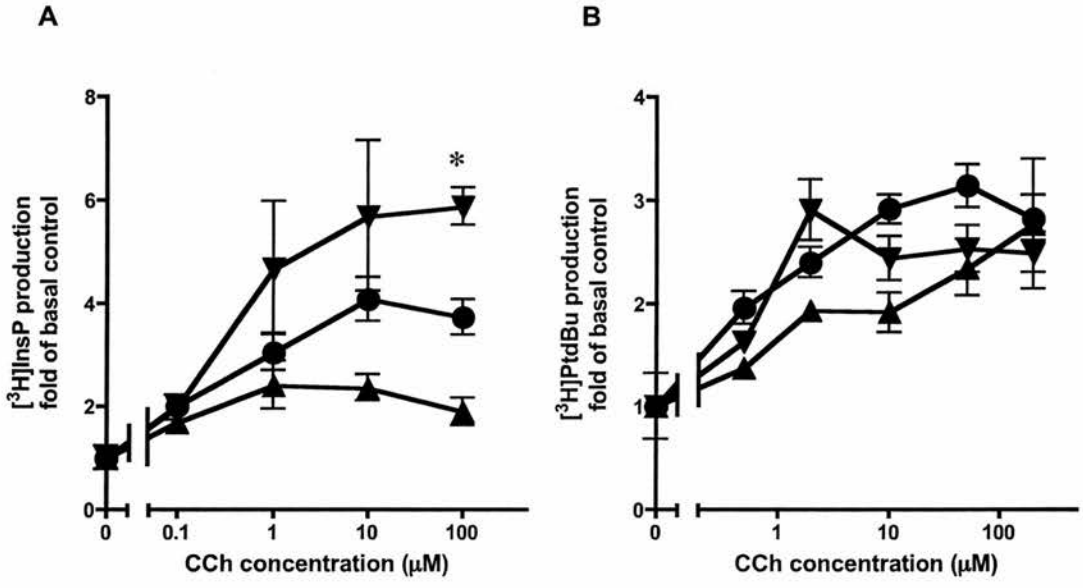
The 14-3-3ζ isoform has a phosphorylation site on threonine-233, which allows negative regulatory phosphorylation by casein kinase 1α (Dubois *et al.*, 1997). Phosphorylation of this residue has been demonstrated to negatively regulate 14-3-3ζ association with c-Raf (Rommel *et al.*, 1996). A mutation of this site from threonine (T) to aspartate (D) is expected to confer surrogate phosphothreonine (233)-like behaviour onto 14-3-3ζ and therefore should reduce 14-3-3ζ affinity for target proteins containing the RSxpSxP motif (Dubois *et al.*, 1997). The effects of the expression of wild type 14-3-3ζ and mutant T233D 14-3-3ζ isoforms on agonist-stimulated M_3 muscarinic receptor phospholipase C and phospholipase D signalling characteristics were investigated. COS 7 cells were transiently transfected with sFM_3 and wild type 14-3-3ζ-myc, mutant T233D 14-3-3ζ-myc or empty vector (as a negative control) cDNAs in a 1:1 ratio and seeded into 12 well plates. The muscarinic agonist carbachol was used in all assays, with a constant stimulation time of 20 minutes. For PLC responses, the cells were labelled with [^{3}H]inositol and serum-deprived for 16 hours. For PLD responses, the cells were labelled with [^{3}H]palmitate and serum-deprived for 16 hours. The [^{3}H]inositol phosphate and [^{3}H]phosphatidylbutanol products were assayed in response to carbachol stimulation and the results are shown in Figure 3.8.

The PLC response to carbachol was attenuated by the overexpression of wild type 14-3-3ζ compared to the control COS 7 cells, which was significant when compared with the overexpression of T233D 14-3-3ζ at concentrations of 100 μM carbachol ($p<0.05$, Wilcoxon test; n=4). The overexpression of either 14-3-3ζ types, did not significantly affect the signalling of PLD compared to control levels ($p>0.05$, Wilcoxon test; n=4).

Any evidence for a specific modulation of one M_3-coupled signalling pathway (PLC) compared to another (PLD), would suggest that 14-3-3 could be involved functionally. One basis for such an influence may be that overexpression of 14-3-3

interferes somehow with the normal expression of the M_3 receptor at the cell surface. However, because the PLD responses are not affected by 14-3-3 expression while PLC responses are attenuated, this may be unlikely to be the mechanism of modulation. Another possibility is that 14-3-3ζ expression could potentially exert a differential influence, as 14-3-3 proteins have been shown to interact with the regulators of G protein signalling (RGS) proteins and have been proposed to negatively regulate their GTPase-activating properties (Benzing *et al.*, 2000; Benzing *et al.*, 2002; Niu *et al.*, 2002). Modulation of the RGS function has been shown to act as a 'molecular switch' for the facilitation of one M_3 coupled pathway over another (Rumenapp *et al.*, 2001). Therefore, if the wild type 14-3-3ζ isoform could negatively regulate an RGS protein involved in controlling the coupling of the M_3 receptor to the Gq heterotrimeric G protein and PLC pathway, overexpression of the wild type 14-3-3ζ might yield a selective attenuation of that pathway. The T233D 14-3-3ζ mutant, which may have a reduced affinity for RGS interaction, might therefore not regulate the RGS protein to the same extent and so may lack any influence exerted by the wild type.

Figure 3.8



14-3-3ζ wild type and mutant effects on PLC and PLD responses of COS 7 cells.

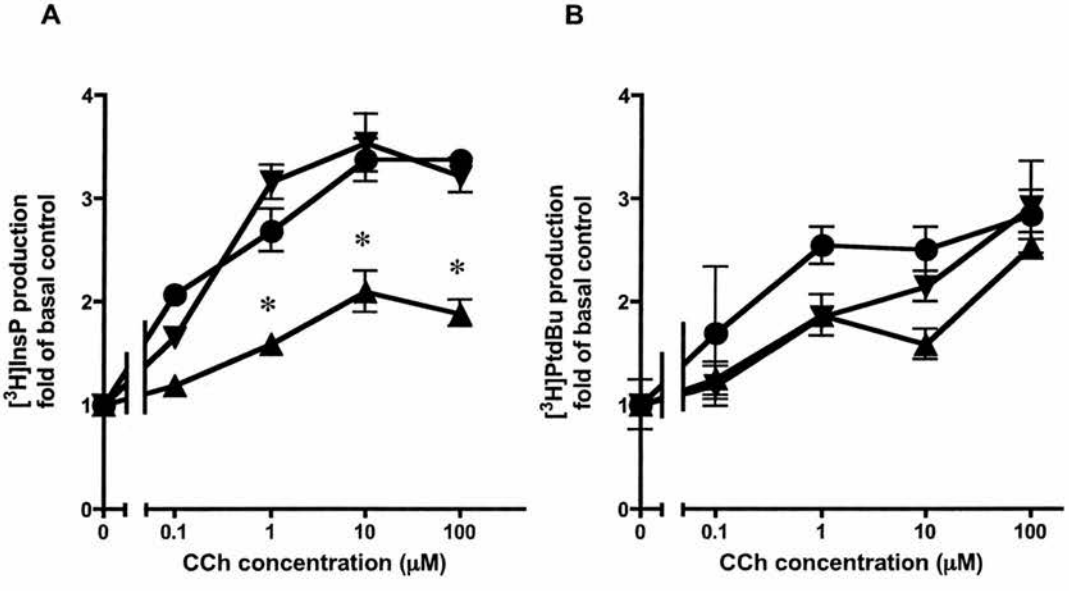
COS 7 cells, transiently transfected with empty control vector (●), wild type 14-3-3ζ-myc (▲) or T233D 14-3-3ζ-myc (▼) were stimulated for 20 minutes with various concentrations of the M_3 agonist carbachol (CCh). The PLC-mediated accumulation of radiolabelled inositol phosphates (A) or the PLD-mediated accumulation of radiolabelled phosphatidylbutanol (B) were measured. The overexpression of wild type 14-3-3ζ caused an attenuation of PLC responses to M_3 agonist, which was significant at concentrations of 100 μM CCh (*$p<0.05$, Wilcoxon test) compared to the T233D 14-3-3ζ mutant (n=4). Overexpression of either of the 14-3-3ζ forms did not significantly affect PLD responses to M_3 agonist ($p>0.05$, Wilcoxon test; n=4).

A putative dimerisation-deficient 14-3-3ε mutant affects the agonist-stimulated properties of the M_3 muscarinic receptor

14-3-3 dimers are formed by interactions of the amino-terminal domains of each monomer (residues 5-21 and 58-89) (Liu *et al.*, 1995; Xiao *et al.*, 1995). Deletion of residues in this domain has been shown to disrupt efficient dimer formation (Jones *et al.*, 1995b). A truncation mutant of the first twenty six amino acid residues of the 14-3-3ε isoform, Δ⁻26 14-3-3ε, was used to investigate the potential signalling implications of dimer disruption in the context of the PLC and PLD signalling pathways of COS 7 cells. The cDNAs of the sFM_3 receptor and wild type 14-3-3ε-myc, mutant Δ⁻26 14-3-3ε-myc, or empty vector as a control, were transiently transfected into COS 7 cells in a 1:1 ratio. The cells were labelled with [^{3}H]inositol and serum-deprived for 16 hours for PLC assays or labelled with [^{3}H]palmitate and serum-deprived for 16 hours for PLD assays. The [^{3}H]inositol phosphate and [^{3}H]phosphatidylbutanol products were assayed in response to 20 minutes of carbachol stimulation and the results are shown in Figure 3.9. The PLC responses to carbachol were significantly attenuated (between the concentrations of 1-100 μM) with wild type 14-3-3ε overexpression ($p<0.05$, Wilcoxon test), but not the Δ⁻26 14-3-3ε mutant, which exhibited no difference from control levels (n=4). For PLD responses, the overexpression of both wild type and Δ⁻26 14-3-3ε isoforms did not significantly affect PLD response to the M_3 agonist ($p>0.05$, Wilcoxon test, n=4).

Similarly to the results with the 14-3-3ζ isoform, a likely candidate target for 14-3-3ε in attenuating carbachol-induced PLC activation may be an RGS protein. The dimerisation deficient mutant of 14-3-3ε, Δ⁻26 14-3-3ε, may not be able to assemble as a stable heterodimer, and the lack of any equivalent inhibitory effect suggests that dimer assembly is necessary for 14-3-3 function here, as proposed by others in different situations (Jones *et al.*, 1995a; Tzivion *et al.*, 1998; Aitken, 2002).

Figure 3.9



14-3-3ε wild type and mutant effects on PLC and PLD responses of COS 7 cells.

COS 7 cells, transiently transfected with empty control vector (●), wild type 14-3-3ε-myc (▲) or Δ⁻26 14-3-3ε-myc (▼) were stimulated for 20 minutes with various concentrations of the M_3 agonist carbachol. The PLC mediated accumulation of radiolabelled inositol phosphates (A) or the PLD mediated accumulation of radiolabelled phosphatidylbutanol (B) were measured. The overexpression of wild type 14-3-3ε caused a significant attenuation of the PLC response to M_3 agonist stimulation (*$p<0.05$, Wilcoxon test), which was mitigated by the overexpression of the Δ⁻26 14-3-3ε mutant (n=4). The overexpression of both wild type 14-3-3ε and Δ26 14-3-3ε caused a slight but not significant attenuation of the PLD response to agonist ($p>0.05$, Wilcoxon test; n=4).

The lack of significant attenuation of the carbachol-induced PLD response by overexpression of both wild type 14-3-3ε and Δ^-26 14-3-3ε indicates that the mechanism used by 14-3-3ε to attenuate M_3 receptor-mediated PLC response is likely to be distinct from that used by the receptor for activation of PLD. This concurs with previous evidence that the mechanism of M_3 receptor mediated PLC and PLD activation are largely distinct in COS 7 cells (Mitchell *et al.*, 2003). An important point that should be considered when interpreting these observations is that one isoform of 14-3-3 may potentially interrupt the usual modulation of certain signalling events by another isoform by functioning as a competitive binding partner or even by forming less effective heterodimers.

Summary

In summary, these experimental findings have shown that 14-3-3 isoforms can associate with the M_3 muscarinic receptor both in GST-fusion studies and in co-immunoprecipitation studies. With the exception of 14-3-3ζ, the isoforms were found to bind to both the M_3 receptor third intracellular loop and carboxy-terminal tail domains with apparently equivalent affinity, suggesting that the site of interaction may be composed of contributions from each of these domains. The specific association of all 14-3-3 isoforms tested with the M_3 receptor, demonstrated by co-immunoprecipitation, indicates that 14-3-3 proteins may associate with the M_3 receptor similarly to the α_2-adrenergic and $GABA_B$ receptors (Prezeau *et al.*, 1999; Couve *et al.*, 2001). However, the precise functional significance of this is not yet clear. The 14-3-3 proteins have been shown to recognise the c-Raf-like phosphoserine motif RSxpSxP or RxY/FxpSxP (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The closest M_3 receptor sequences as candidates for the 14-3-3 recognition site, are the residues RSCSSYEL (288-295) or TATLP (451-455) within the third intracellular loop domain (Obenauer *et al.*, 2003). However, the likely interaction site would need to be more thoroughly investigated by using mutagenesis studies involving the third intracellular loop and carboxy-terminal tail domains of the M_3 receptor. Furthermore, as membrane-associated

14-3-3 is primarily found at the plasma membrane, this indicates that a functional assembly of the M_3 receptor and 14-3-3 isoforms could potentially occur *in vivo* and may implicate 14-3-3 in the signalling of GPCRs.

The nature of wild type (but not mutant) 14-3-3ε potentially influencing the whole-cell signalling characteristics of the M_3 muscarinic receptor activation of PLC, but not PLD, implies that there may be a modulation of a protein involved in the pathway. A likely candidate for this is the RGS protein, involved in coupling the heterotrimeric G protein to the PLC pathway, as 14-3-3 has been shown to interact with these signalling regulators (Benzing *et al.*, 2000; Benzing *et al.*, 2002). This may also be the case with the modulation of signalling by the wild type 14-3-3ζ isoform, which displayed a similar inhibitory trend when compared to the T233D 14-3-3ζ mutant. If the RGS proteins can influence the signalling characteristics within the cell by facilitating or impeding G protein activation (Berman and Gilman, 1998; Rumenapp *et al.*, 2001), the modulation of specific RGS protein activity by 14-3-3 proteins (in an isoform dependent manner) may present a mechanism of regulation that is consistent with the results found in this chapter. Further investigations into whether the RGS proteins are involved (for example whether a specific type of RGS protein is associated with 14-3-3 upon carbachol stimulation or whether the RGS/14-3-3 may exist as a complex with the M_3 receptor) would need to be made, for example in the use of siRNA or specific blockade of the interface for 14-3-3 binding by decoy peptides.

Furthermore, it has been previously shown that the product of PLD activity, phosphatidic acid, is an important cofactor for the activation of the MAP kinase pathway (Rizzo *et al.*, 1999; Andresen *et al.*, 2002; Ghosh *et al.*, 2003) and the M_3 receptor has been shown to couple to the MAP kinase signalling pathway upon agonist stimulation and desensitisation (Budd *et al.*, 1999). As 14-3-3 proteins have already been demonstrated to be important in the Ras and PKC mediated activation of c-Raf (and subsequent MAP kinase activation) (Fantl *et al.*, 1994; Freed *et al.*, 1994), it may be possible that 14-3-3 proteins are

involved in the M_3 receptor-mediated (and possibly PLD-dependent) activation of the MAP kinase pathway. Following activation of the M_3 receptor, the subsequent involvement of PKC-phosphorylated c-Raf may compete for M_3:14-3-3 interaction and allow the receptor to undergo another series of critical signalling or internalising interactions. Further investigations into the role of both 14-3-3 and PLD in the potential facilitation of MAP kinase activation may be very valuable in understanding the coordination of parallel signalling pathways (signal crosstalk).

The physiological implications of the role of 14-3-3 in the signal transduction characteristics of the M_3 receptor remain unclear. The interaction of 14-3-3 isoforms with the receptor, as demonstrated in this chapter, may imply that 14-3-3 provides a scaffolding role for the receptor. However, the potential modulation by 14-3-3 of the effector responses to agonist stimulation of the M_3 receptor also implies that 14-3-3 may have distinct functional roles in modulating the signalling mechanism of the M_3 receptor.

Chapter 4:

The interaction of PLD with the M_3 muscarinic receptor

Introduction

The potential for a phospholipase D to interact directly with a G protein-coupled receptor (GPCR) was identified when the amino terminal domain of PLD2 was found to interact with the carboxy-terminal tail domain of the μ-opioid receptor in a yeast two-hybrid screen (Koch *et al.*, 2003). The PLD2 (but not PLD1) isozyme could further be co-immunoprecipitated with the μ-opioid receptor in a constitutive manner and was demonstrated to be necessary for the agonist-dependent internalisation of the receptor. Phosphatidic acid produced by PLD2 activity was proposed to recruit the AP-2 adapter complex and the clathrin coat protein to the membrane for the formation of endocytotic vesicles, which were necessary for receptor internalisation. Inhibition of PLD activity resulted in an inhibition of receptor endocytosis (Koch *et al.*, 2003; Koch *et al.*, 2004). The GPCR interacting domain within PLD2 was determined to be in the amino-terminal region of the isozyme, which contains a Phox homology (PX) domain. As PLD1 also contains a conserved PX domain, it is interesting to question why isozyme specificity for PLD2 was observed in that study. Furthermore, PLD2 rather than PLD1 has been implicated in the endocytic internalisation of the angiotensin II type 1A (AT_{1A}) receptor. However the possibility of a direct interaction between the receptor and PLD2 was not addressed (Du *et al.*, 2004). It was therefore important to question whether PLD could interact with the M_3 muscarinic receptor as found with other GPCRs and whether any interaction was isozyme specific. In this chapter the strategies of GST-fusion pull-downs and co-immunoprecipitation were used to determine whether PLD could interact with the M_3 muscarinic receptor.

Basal endogenous PLD levels are low within most cells and PLD activity is only transiently elevated upon stimulation (Exton, 1994; Cockcroft, 1996; Cockcroft, 2001). This has led to a difficulty in elucidating the signalling role of PLD and has necessitated using epitope tagged PLD constructs in the determination of PLD localisation and function. The

subcellular localisation of PLD isozymes has been investigated by a number of different groups (Chen *et al.*, 1997; Colley *et al.*, 1997; Brown *et al.*, 1998; Czarny *et al.*, 1999; Czarny *et al.*, 2000; Freyberg *et al.*, 2001; Sarri *et al.*, 2003; Du *et al.*, 2004). In the majority of these studies, the PLD2 isozyme has been characterised as being associated almost exclusively with the plasma membrane of most cell types under resting conditions (Colley *et al.*, 1997; Czarny *et al.*, 2000; Cockcroft, 2001; Du *et al.*, 2004). A recent report using high affinity antibodies considered to selectively recognise endogenous PLD2 has suggested that it is present at high levels in the Golgi apparatus, with only a small proportion present at the plasma membrane (Freyberg *et al.*, 2002). This localisation, however, remains disputed, as endogenous PLD2 activity has also been demonstrated primarily at the plasma membrane by others (Du *et al.*, 2004).

The distribution of PLD1 appears to be quite divergent in different cell types. The majority of reports indicate that PLD1 localises mainly to intracellular membranes such as the Golgi apparatus, endosomes and lysosomes in most resting cells (Ktistakis *et al.*, 1995; Chen *et al.*, 1997; Cockcroft, 2001). Investigations made by Freyberg and colleagues, using highly selective antibodies for PLD1 have shown that endogenous PLD1 is present in the Golgi apparatus and even in the nuclear compartment (Freyberg *et al.*, 2001). In PC12 cells however, PLD1 associates mainly with the plasma membrane (Vitale *et al.*, 2001; Du *et al.*, 2003) and there are also reports of PLD1 activity at the plasma membrane (as well as intracellular membranes) in other cell types (Kim *et al.*, 1999; Lucocq *et al.*, 2001). The ability of PLD to localise to particular membranes is thought to be conferred by the lipid-interacting domains. The pleckstrin homology (PH) domain has been shown to be important for PLD localisation to membranes, as it interacts with PIP_2 (although this particular interaction is not necessarily involved in PLD activation) (Hodgkin *et al.*, 2000; Du *et al.*, 2003). Upon cellular stimulation, it has been shown that PLD1 translocates from intracellular membrane compartments to the plasma membrane (Morgan *et al.*, 1997; Brown

et al., 1998; Du *et al.*, 2003; Mitchell *et al.*, 2003). It has been proposed that this translocation and subsequent recycling from the plasma membrane to the intracellular compartment may involve a series of sequential interactions of the different conserved lipid-interacting domains with lipid membranes (Du *et al.*, 2003).

In order to substantiate and develop previous results from subcellular fractionation (Mitchell *et al.*, 2003) it was important to visualise the expression characteristics of PLD isoforms in the COS 7 cell system. The technique of confocal microscopy was used to determine the subcellular localisation of the M_3 receptor and PLD isozymes in COS 7 cells and to observe the effect of muscarinic receptor agonist stimulation on these localisations.

The phospholipase D signalling ability of the M_3 muscarinic receptor through both the classical heterotrimeric G-protein activated pathway and ADP-ribosylation factor (ARF) activated (or other small G protein-dependent) pathways has been investigated previously (Rumenapp *et al.*, 1995; Schmidt *et al.*, 1995; Schmidt *et al.*, 1997; Mitchell *et al.*, 1998). The receptor-activated ARF-dependent component of PLD signalling is sensitive to the ARF-guanine nucleotide exchange factor (ARF-GEF) inhibitor brefeldin A (BFA) (Donaldson *et al.*, 1992; Guillemain and Exton, 1997; Mitchell *et al.*, 1998). Therefore the contribution of the receptor-mediated ARF-dependent component of PLD activity, can be calculated from the relative reduction in the agonist activated response in the presence of BFA (Mitchell *et al.*, 1998). Furthermore, the demonstration that a dominant negative mutant of PLD1 (K898R), but not PLD2 (K758R), can partially inhibit the PLD response to the M_3 agonist carbachol indicates that in COS 7 cells, the PLD1 isozyme is the primary mediator of the receptor stimulated PLD response (Mitchell *et al.*, 2003).

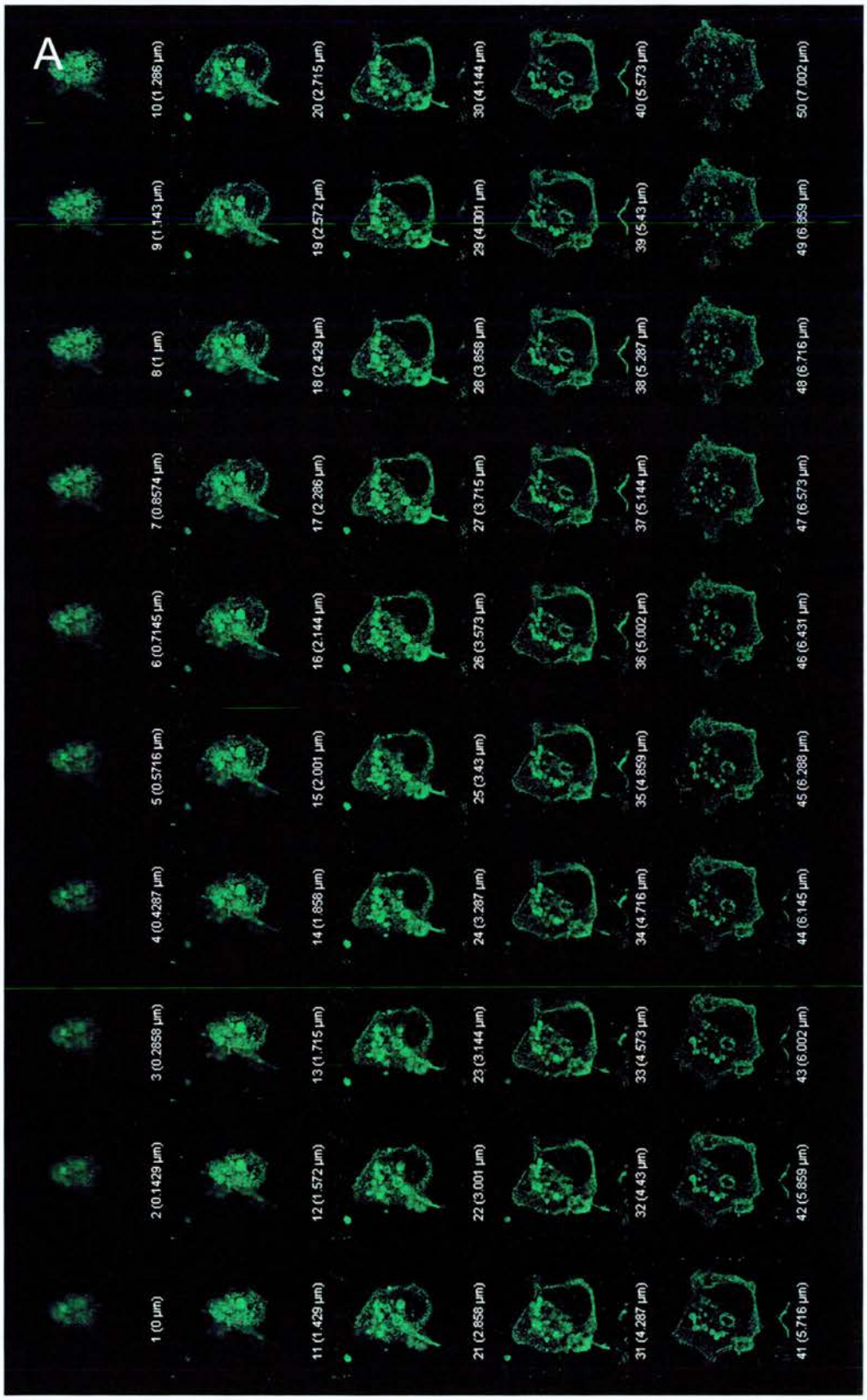
Confocal immunofluorescence imaging of PLD

Visualising the basal localisation of PLD was carried out by confocal immunofluorescence imaging after transfecting COS 7 cells with HA tagged PLD1 or PLD2. The cells were serum-deprived for 6 hours, permeabilised and fixed overnight. The fixed cells were stained with primary mouse monoclonal anti-HA antibody clone 12CA5 (Roche Diagnostics, East Sussex, UK) followed by Alexa Fluor goat anti-mouse (488 nm) secondary antibody (Molecular Probes, Leiden, The Netherlands). The cells were then imaged by confocal microscopy, acquiring data at Nyquist sampling rates. Healthy (non-rounded) transfected cells were selected at random and were scanned equivalently using identical experimental conditions throughout image acquisition (maintaining both laser power and photon-multiplier tube amplification in software). The Nyquist sample rate is defined as the minimum theoretical sampling rate to fully describe a band limited signal; for a maximum frequency component of f_0 the Nyquist rate would need to at be least $2f_0$ (for a wavelength of 488 nm the values were typically an xy-field of 47 x 47 nm and z-step of 130 nm at 1024 x 1024 pixel resolution).

Confocal images of unstimulated COS 7 cells expressing HA-PLD1 and HA-PLD2 are shown in Figure 4.1 and Figure 4.2 respectively. The localisation of PLD2 in COS 7 cells is mainly associated with the plasma membrane, consistent with most other findings (Colley *et al.*, 1997; Czarny *et al.*, 2000; Sarri *et al.*, 2003; Du *et al.*, 2004). There was minimal PLD2 antibody staining of intracellular compartments, such as the Golgi apparatus, which was observed by Freyberg and colleagues (Freyberg *et al.*, 2002). In contrast to that report, our findings with HA-tagged PLD2 expressed in COS 7 cells yielded results entirely consistent with most other studies showing a predominant plasma membrane localisation.

In COS 7 cells, PLD1 was localised mainly to the perinuclear regions, presumed to be the Golgi apparatus, in accordance with other findings (Brown *et al.*, 1998; Cockcroft, 2001) with an additional smaller component present at the plasma membrane, which has

Figure 4.1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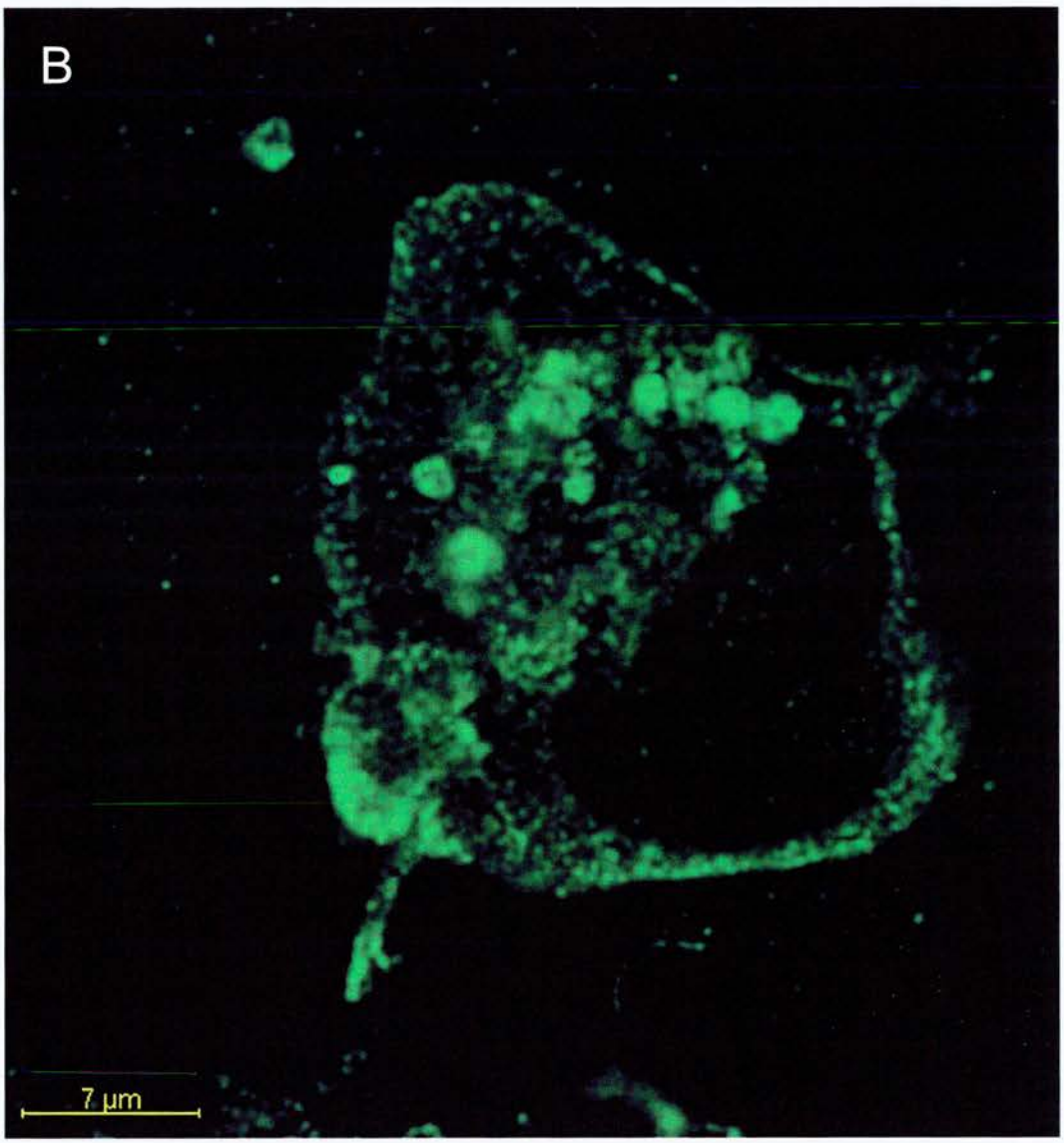
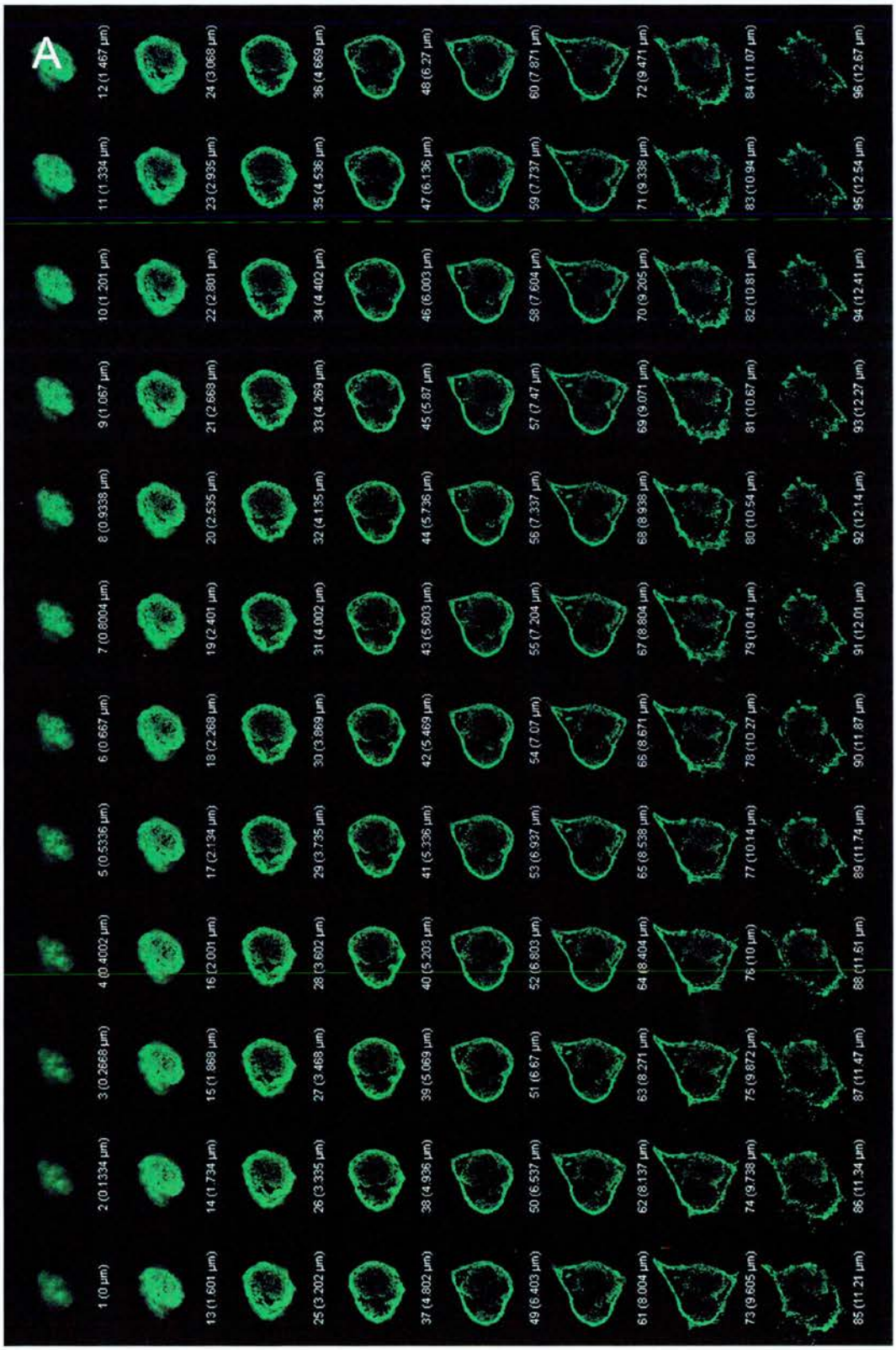


The localisation of PLD1 in unstimulated COS 7 cells.

The gallery (A) and mid-section (B) views of an unstimulated COS 7 cell, transiently transfected with HA-PLD1 and stained for the HA tag with AlexaFluor 488 nm antibody (green). The PLD1 isoform consistently localises predominantly to punctate intracellular and perinuclear structures with a small proportion localising with the plasma membrane.

Figure 4.2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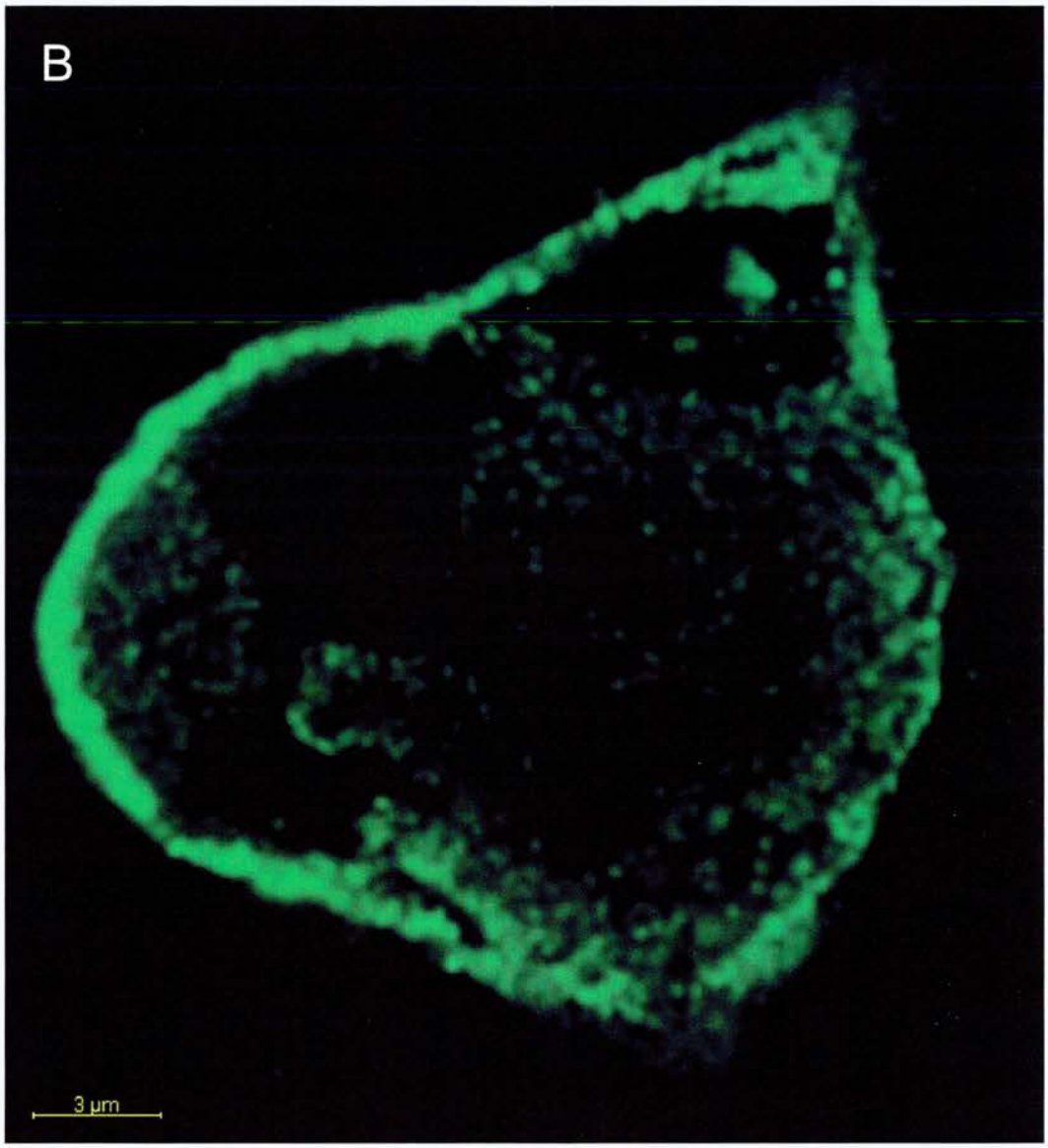


The localisation of PLD2 in unstimulated COS 7 cells.

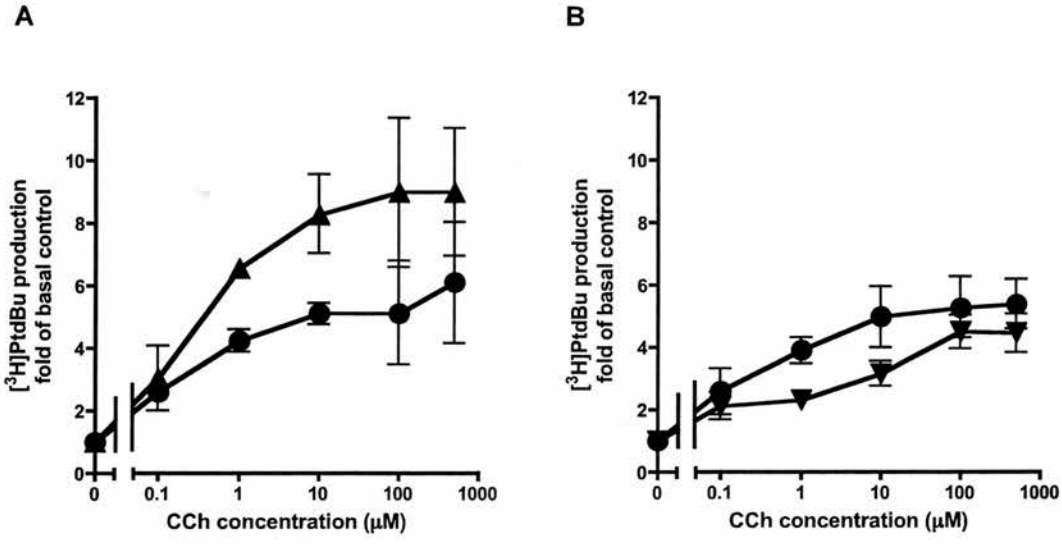
The gallery (A) and mid-section (B) views of an unstimulated COS 7 cell, transiently transfected with HA-PLD2 and stained for the HA tag with AlexaFluor 488 nm antibody (green). The PLD2 isoform consistently localises to the plasma membrane in agreement with other findings.

been observed by some others (Lucocq *et al.*, 2001). It is possible that the large vesicular-type structures visualised may be indicative of the expression of the transfected PLD compared to endogenous PLD levels (Freyberg *et al.*, 2001). In a previous study by our group, endogenous PLD1 was found mainly on the intracellular compartment membranes, with little presence at the plasma membrane in unstimulated COS 7 cells (Mitchell *et al.*, 2003). In the Freyberg study, endogenous PLD1 was localised to the perinuclear and nuclear regions, but there was also some endogenous PLD1 present at the plasma membrane (Freyberg *et al.*, 2001). This suggests that the partial localisation of transfected HA-PLD1 to the plasma membrane is a good reflection of the distribution seen normally with native PLD1.

Signalling of the M_3 receptor to PLD

It has been proposed that full activity of PLD1 requires the synergistic co-activation by ARF, Rho and PKC together (Kuribara *et al.*, 1995; Hodgkin *et al.*, 1999). The PLD1 mutant PIM87/I870R (PIM/IR HA-PLD1) construct has mutations in the residues that are thought to be important for the activation of PLD1 by RhoA (a mutation of isoleucine 870 to arginine) and PKC (an insertion of GVPLE at position 87) (Yamazaki *et al.*, 1999; Zhang *et al.*, 1999). The mutant can be thought of as being only ARF responsive (Du *et al.*, 2000). Signal FLAG-tagged M_3 receptor and either empty vector, wild type HA-PLD1 or the PIM/IR HA-PLD1 mutant were transiently expressed in COS 7 cells and the PLD response to the muscarinic M_3 receptor agonist, carbachol, was measured in a concentration-dependent manner. The log concentration-response curves to carbachol for the wild type PLD1 and the PIM/IR PLD1 mutant are shown in Figure 4.3.

Figure 4.3



The contribution of direct ARF dependent PLD activation in COS 7 PLD responses.

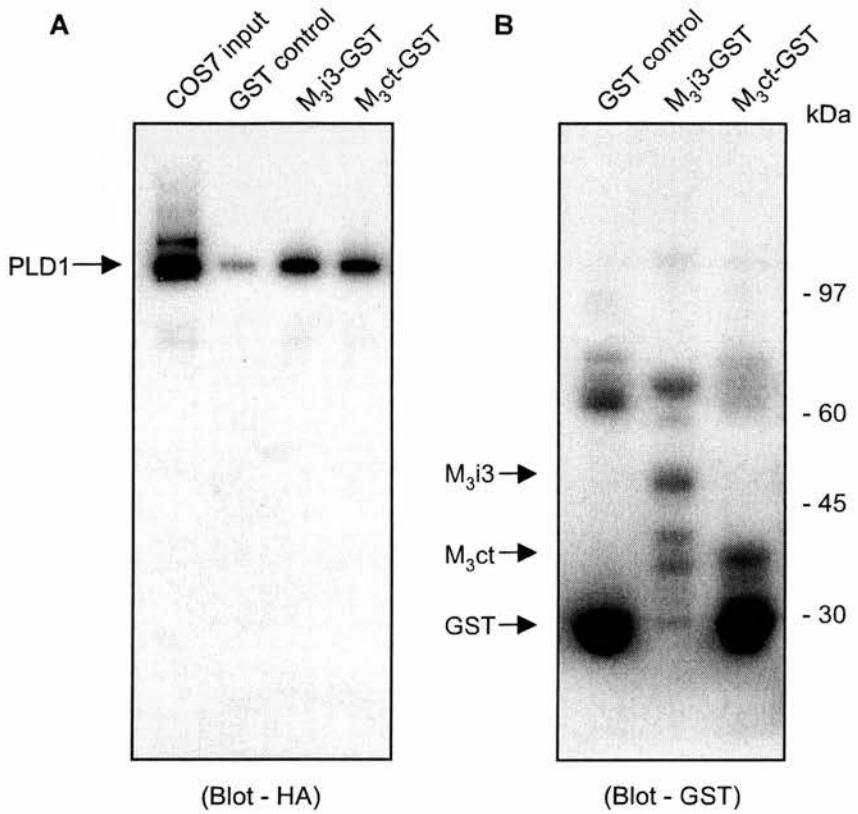
COS 7 cells were transiently transfected with sFM_3 receptor and empty vector (●), sFM_3 and wild type HA-PLD1 (▲) (A) or sFM_3 receptor and the ARF only responsive PLD1 mutant HA-PIM87/I870R (▼) (B). The cells were stimulated for 20 minutes with different concentrations of the M_3 receptor agonist carbachol (CCh) and the log-concentration response curves are shown. In (A), non-linear regression analysis indicated that the mean EC_{50} values for CCh in wild type PLD1 and control vector transfected cells were 0.35 and 0.23 μM respectively (n=4). The mean maximal response to CCh in wild type PLD1 transfected cells was increased to 162 ± 18.2% of that in control ($p>0.05$, Wilcoxon test). In (B), non-linear regression analysis indicated that the mean EC_{50} values for CCh in PIM87/I870R PLD1 and control vector transfected cells were 6.86 μM and 0.27 μM respectively (n=4). The mean maximal response to CCh in PIM87/I870R PLD1 cells was 89 ± 5.6% of that in control ($p>0.05$, Wilcoxon test). These findings are consistent with the hypothesis that full M_3 agonist-mediated PLD1 activation arises from a synergistic action of ARF, PKC and also potentially RhoA in COS 7 cells.

Non linear regression analysis indicated that the mean EC_{50} in wild type PLD1-transfected cells was 0.35 μM and for PIM/IR PLD1 was 6.86 μM carbachol - this yielded a dose ratio of approximately 25 fold (n=4) and is consistent with a lower potency of M_3 receptor-evoked PLD1 activation. The mean EC_{50} of M_3 and vector only-transfected cells was 0.23 μM and 0.27 μM carbachol respectively. However, the mean maximal response of the PIM/IR PLD1 mutant was 89 ± 5.6% of control (compared to 162 ± 18.2% in wild type PLD1), indicating that it did not attenuate the response in the way seen with the catalytically inactive PLD1 mutant (K898R-PLD1) (Mitchell *et al.*, 2003). These observations agree with previous evidence that PKC, RhoA and ARF act synergistically to fully activate PLD1 and that each may potentially play a role in M_3 receptor-evoked PLD responses.

M_3 receptor domains interact with PLD in vitro

Glutathione S-transferase (GST) fusion proteins of the third intracellular loop of the M_3 receptor (M_3i3) and the carboxy-terminal tail domain of the M_3 receptor (M_3ct) were prepared as in the previous chapter. These GST fusion proteins and GST as a negative control were immobilised onto glutathione Sepharose 4B to create an affinity matrix. Cellular extract from COS 7 cells overexpressing HA-tagged PLD1 was applied to the prepared affinity matrix and incubated at 4 °C overnight with rolling. Bound proteins were lysed from the beads, separated by SDS-PAGE, and Western blotted. HA-PLD1 levels were visualised with a primary rat monoclonal anti-HA antibody conjugated to horseradish peroxidase (HRP) (Roche Diagnostics) and a representative result from three independent experiments is shown in Figure 4.4A. Input levels of HA-PLD immunoreactivity in extracts were also monitored and both fusion protein and interacting protein inputs were balanced to ensure comparability between samples. The level of GST fusion protein in the input matrix was visualised using primary rabbit polyclonal anti-GST antibody (Santa Cruz), followed by anti-rabbit HRP-conjugated secondary antibody (Chemicon) and is shown in Figure 4.4B.

Figure 4.4



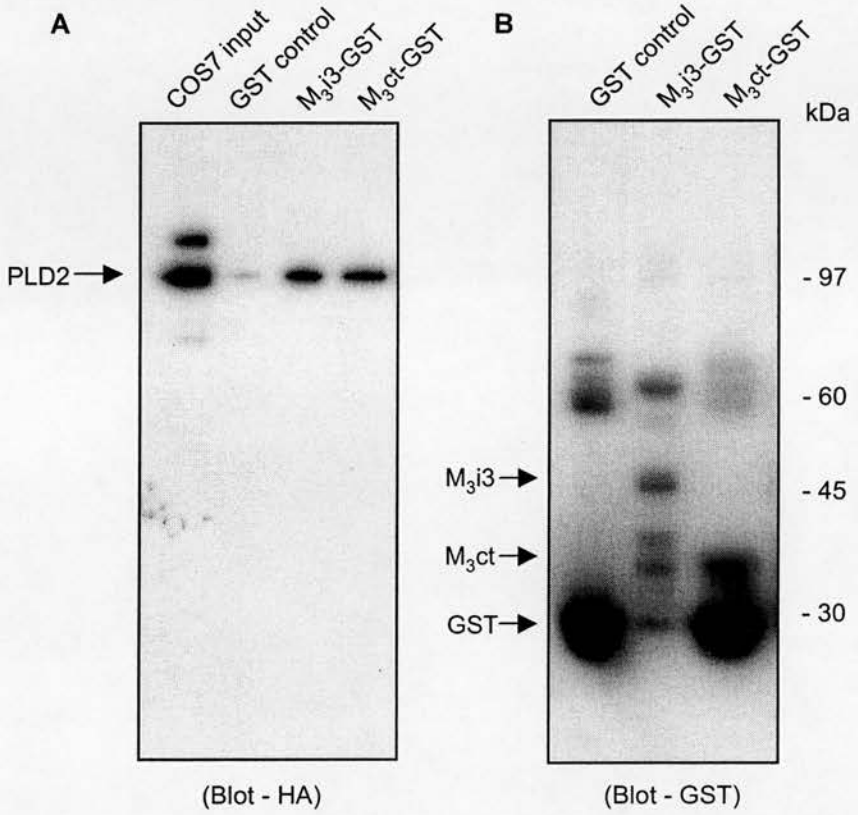
PLD1 associates with the M_3 receptor *in vitro*.

COS 7 cell extracts transiently overexpressing HA-PLD1 were incubated with GST fusion proteins of the M_3 receptor third intracellular loop (M_3i3) and carboxy-terminal tail (M_3ct) domains, and with GST only as a negative control. The associated PLD1 was visualised using anti-HA antibody (A). The GST fusion proteins of the M_3 receptor pull down PLD1 with an equal affinity for both constructs. The GST fusion constructs of the input proteins are also shown, visualised using anti-GST antibody (B). The bands above 60 kDa are probably due to breakdown products in the bacterial expression system.

The expression of the GST-fusion construct of the M_3ct was consistently accompanied by a high level of expression of GST alone. Nevertheless, inputs were balanced for the band of the correct molecular mass for the complete GST-M_3ct construct and because the levels of PLD association with GST control were so low, it was most unlikely that the contribution of GST itself was a significant factor in the results. The matching of the band density for both the M_3i3 and M_3ct GST-fusion constructs allowed effective comparison of the results. PLD1 appeared to bind to both GST-fusion constructs of the M_3 receptor specifically above GST control levels and with apparently similar affinity, suggesting that any interaction of PLD1 with the receptor *in vivo* may be dependent upon a molecular mechanism involving both the third intracellular loop and carboxy-terminal tail domains.

The *in vitro* GST-fusion binding assay was repeated, using cell extracts from HA-PLD2 transfected COS 7 cells and incubating with GST-M_3i3, GST-M_3ct and GST only, as a negative control. The associated proteins were lysed, separated by SDS-PAGE, Western blotted and visualised as before. The PLD2 associating with the affinity matrices is shown in Figure 4.5A, with the input levels of GST-fusion constructs in Figure 4.5B (and is a representative result from three independent experiments). PLD2, as PLD1, bound to both M_3 constructs specifically, and also with an apparently similar affinity for both. These results contrast with the findings made by Koch and colleagues in the μ-opioid receptor, where the PLD2 isozyme was found to interact with the carboxy-terminal tail domain of the receptor and PLD1 was not found to interact at all (Koch *et al.*, 2003). Evidence that the interaction of PLDs with GST-fusion proteins is dependent on domain amino acid sequence and not non-specific receptor interaction has been provided by other studies in the laboratory (where PLD1 has been shown to bind very selectively to the carboxy-terminal domain but not the third intracellular loop of the 5-HT_{2A} GPCR). The results of the GST-fusion protein studies here imply that the molecular interactions underpinning the association of both PLD1 and PLD2 to the M_3 receptor *in vitro* are largely equivalent and

Figure 4.5



PLD2 associates with the M_3 receptor *in vitro*.

COS 7 cell extracts transiently overexpressing HA-PLD2 were incubated with GST fusion proteins of t`he M_3 receptor third intracellular loop (M_3i3) and carboxy-terminal tail (M_3ct) domains, and with GST only as a negative control (visualised with anti-HA antibody) (A). The GST fusion proteins of the M_3 receptor pull down PLD2 with an equal affinity for both constructs. The GST fusion constructs of the input proteins are also shown, visualised with anti-GST antibody (B).

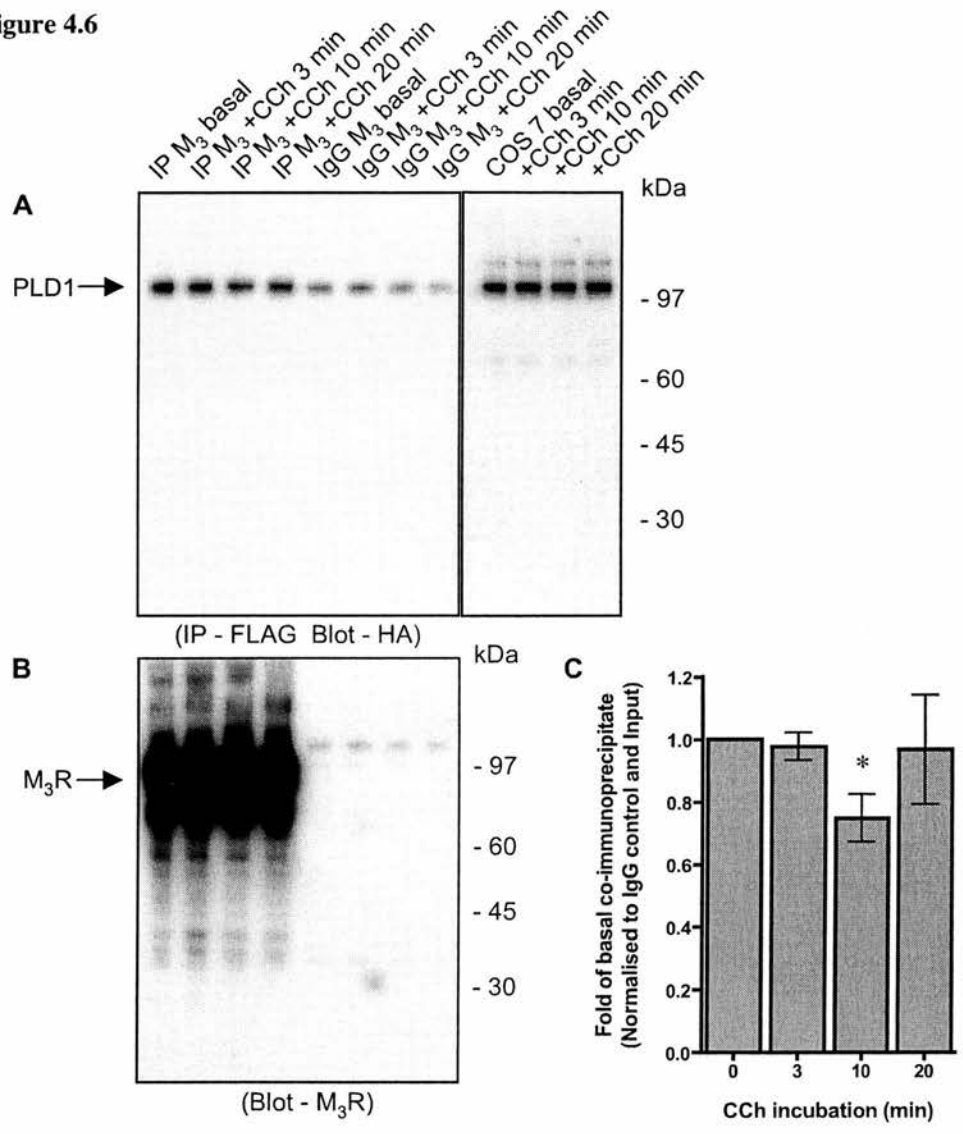
are not isozyme dependent in this system. We therefore carried out further co-immunoprecipitation experiments to determine whether equivalent results were obtained under more physiological conditions.

PLD1 co-immunoprecipitates with the M_3 receptor

COS 7 cells were transiently transfected with signal FLAG tagged M_3 receptor and HA tagged PLD1 cDNAs in a 1:1 ratio. Cells were stimulated where necessary with 100 μM of the M_3 receptor agonist, carbachol, for 3, 10 and 20 minutes prior to lysis. Immunoprecipitates directed against the FLAG tag were prepared by incubating precleared cellular supernatants with 3 μg/ml mouse anti-FLAG antibody clone M2 (Sigma Aldrich, Dorset, UK) or 3 μg/ml non-immune mouse IgG as a control, followed by 40 μl/ml of a 1:1 suspension of Protein-G Sepharose in immunoprecipitation buffer. The beads were washed and co-immunoprecipitates were lysed from the matrix before the proteins were separated by SDS-PAGE, Western blotted and visualised using rat anti-HA HRP-conjugated primary antibody (Roche Diagnostics). The levels of PLD1 that were co-immunoprecipitated with the M_3 receptor are shown in Figure 4.6A. The levels of directly immunoprecipitated M_3 receptor were visualised with anti-M_3 receptor rabbit polyclonal primary antibody (kindly gifted by Dr A. Tobin), followed by anti-rabbit HRP-conjugated secondary antibody and are shown in Figure 4.6B.

The PLD1 isozyme was found to specifically co-immunoprecipitate with the M_3 receptor under basal conditions. This association was transiently and consistently reduced upon agonist stimulation of the M_3 receptor where it was at a minimum of approximately 75% ± 10% (n=5) of basal association at the 10 minute time point. This reduction was statistically significant ($p<0.05$, Unpaired student t-test) (Figure 4.6C). After 20 minutes of carbachol stimulation, the level of PLD1 co-immunoprecipitating with the M_3 receptor was no different to basal levels. One explanation for this decrease may be that the agonist-

Figure 4.6



PLD1 co-immunoprecipitates with the M_3 receptor.

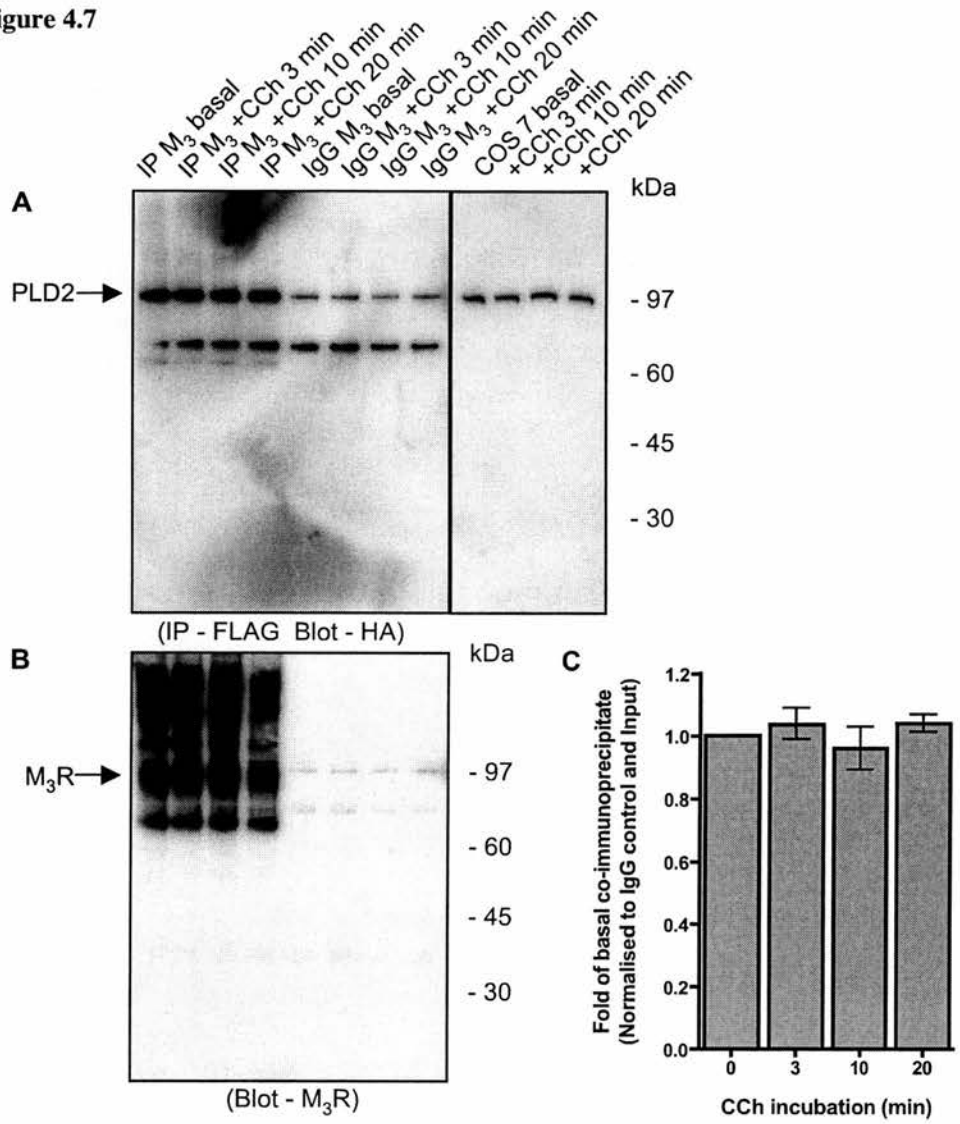
COS 7 cells were transiently transfected with sFM_3 receptor and HA-PLD1. The cells were stimulated with 100 μM carbachol (CCh) for the times indicated. Immunoprecipitates were directed against the FLAG tag of the receptor and co-immunoprecipitated PLD1 levels visualised by immunoblotting against the HA tag (A). The levels of FLAG immunoprecipitate, probed with anti-M_3 antibody (A. Tobin), are shown in (B). PLD1 co-immunopecipitates with the M_3 receptor and the association is significantly reduced upon M_3 agonist stimulation at 10 min (C) (n=5).

induced stimulation of the M_3 receptor causes dissociation of PLD1 from the receptor due to the activation state of PLD (i.e. that it exists as a complex in a basal state) and active PLD1 dissociates from the receptor, re-associating with the receptor as PLD1 is later inactivated. An alternative reason for the dissociation could be that PLD1 may facilitate agonist stimulated endocytosis of the M_3 receptor as is the case with the μ-opioid receptor (Koch *et al.*, 2003). In this model, it may be the case that PLD1 facilitates the internalisation of the receptor, resulting in the dissociation of PLD1 from the receptor after the receptor has been successfully sequestered away from the plasma membrane. Indeed, Koch and colleagues noted that the facilitation of the μ-opioid receptor internalisation by PLD2 resulted in a dissociation of the PLD2 isozyme from the receptor after 30 minutes of agonist stimulation (Koch *et al.*, 2003). The internalisation of the M_3 muscarinic receptor is quite swift in COS 7 cells, with the majority of final sequestered receptor binding sites being internalised within 10 minutes of agonist stimulation (Johnson *et al.*, 2004). In addition to this, this translocation of PLD1 to the plasma membrane upon carbachol stimulation has been shown to have taken place at this time point (Mitchell *et al.*, 2003). If the agonist-induced translocation and subsequent dissociation of PLD1 from the receptor is indicative of the active phospholipase D facilitating endocytosis (possibly by the production of phosphatidic acid and the recruitment of clathrin assembly proteins), this would agree with the findings made by Koch and colleagues (Koch *et al.*, 2003). Furthermore, a subsequent report made by Koch and co-workers has demonstrated that PLD may have an additional role in the recycling of the G protein-coupled receptors (Koch *et al.*, 2004). The apparent re-association of PLD1 with the M_3 receptor after 20 minutes of agonist stimulation may be indicative of a similar mechanism of PLD-dependent recycling of the M_3 receptor to cause resensitisation.

PLD2 co-immunoprecipitates with the M_3 receptor

The co-immunoprecipitation experiment was repeated as before, following co-transfection of the M_3 receptor with HA-PLD2 into COS 7 cells. Stimulation was carried out with 100 μM carbachol for 3, 10 or 20 minutes where indicated. Immunoprecipitates directed against the FLAG tag of the receptor were prepared and co-immunoprecipitated proteins were lysed from the beads, separated by SDS-PAGE, Western blotted and visualised as before. PLD2 was also co-immunoprecipitated with the M_3 receptor under basal conditions, however this interaction was not detectably altered following carbachol stimulation (after 10 min stimulation the band density was 95% ± 8% of basal, n=4) (Figure 4.7). This contrasts with the finding made previously with the PLD1 isozyme and implies that any functional role that the dissociation of phospholipase D from the M_3 receptor may reflect some isozyme specific function within the COS 7 system. This difference may be attributed to a number of things. It may be that although PLD2 co-immunoprecipitates with the M_3 receptor it does not have any functional purpose (if dissociation is an indicator of the facilitation of receptor endocytosis). This seems unlikely, as the μ-opioid receptor associated with PLD2 but not PLD1 at all when undergoing endocytosis (Koch *et al.*, 2003). The indication that PLD1 and PLD2 both interact with the receptor, also implies that both may be able to mediate any functional role. It may possibly be that there is a reduction in the association of PLD2 with the M_3 receptor that is not as readily detectable using standard immunodetection methods as for the PLD1 isozyme. The reduction in the levels of PLD1 may be significant, however there may be very small changes in the levels of PLD2 that are too sensitive for immunodetection. The fact that PLD2 is substantially localised to the plasma membrane and is therefore available in greater supply for receptor interaction compared to PLD1 (which has to translocate to the plasma membrane) may mean that any PLD2 dissociating from the receptor is rapidly replaced so that no overall reduction in interaction can be detected. It may also be the case that it is PLD2 that facilitates the endocytosis of the M_3 receptor,

Figure 4.7



PLD2 co-immunoprecipitates with the M_3 receptor.

COS 7 cells were transiently transfected with sFM_3 receptor and HA-PLD2. The cells were stimulated with 100 μM carbachol (CCh) for the times indicated. Immunoprecipitates were directed against the FLAG tag of the receptor and co-immunoprecipitated PLD2 levels visualised by immunoblotting against the HA tag (A). The levels of FLAG immunoprecipitate, probed with anti-M_3 antibody (A. Tobin), are shown in (B). PLD2 co-immunopecipitates with the M_3 receptor and the association is not significantly affected by M_3 agonist stimulation (C) (n=5).

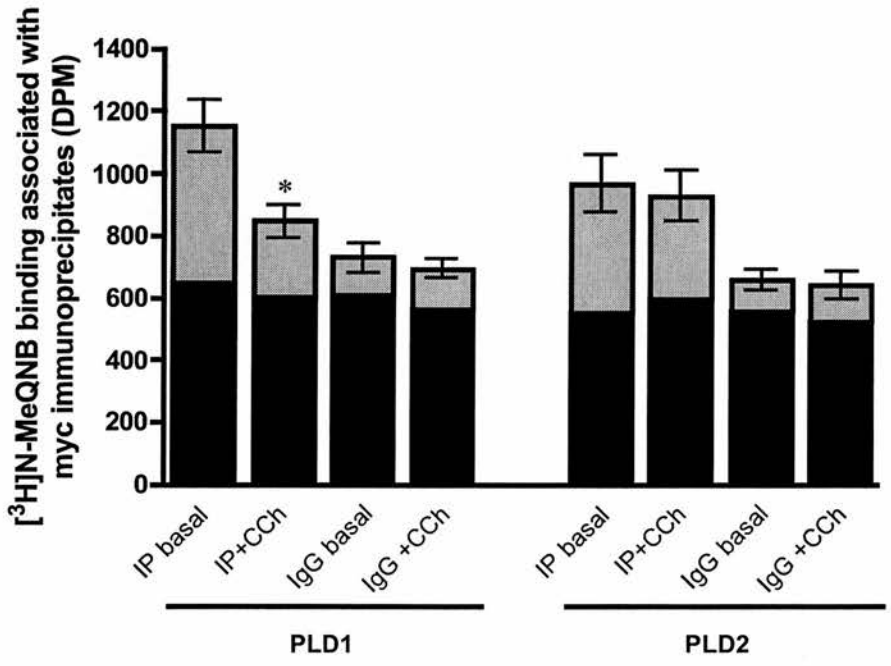
preferentially to the PLD1 isozyme. The continued association of PLD2 may be a more accurate reflection of the facilitation of M_3 receptor endocytosis, compared to the on-off characteristics of the PLD1 association with the receptor.

The M_3 receptor co-immunoprecipitates with PLD1 and PLD2

The interaction of PLD with the M_3 receptor has been demonstrated by immunoblot detection of both PLD1 and PLD2 co-immunoprecipitating with the M_3 receptor. To provide independent corroboration of this interaction, the co-immunoprecipitation of the M_3 receptor with PLDs was investigated using specific [^{3}H]N-Me QNB ligand binding to the M_3 receptor in HA-PLD-directed immunoprecipitates (this experiment was performed by Dr R. Mitchell and Ms M. S. Johnson).

COS 7 cells were transiently transfected with signal FLAG tagged M_3 (sFM_3) receptor and HA-tagged PLD1 or PLD2 (HA-PLD) cDNAs in a 1:1 ratio. The COS 7 cells were stimulated with 200 μM of the M_3 agonist carbachol, where necessary, for 10 minutes before lysis. Immunoprecipitates directed against the HA tag were prepared by incubating precleared cellular supernatants with 2 μg/ml mouse monoclonal anti-HA antibody clone 12CA5 (Roche Diagnostics) or 2 μg/ml non-immune mouse IgG as a control, followed by 40 μl/ml of a 1:1 of Protein-G Sepharose suspension. The washed HA-PLD immunoprecipitates were assayed for the presence of M_3 muscarinic receptor binding sites by incubating the immunoprecipitates with 2.38 nM [^{3}H]N-Me-QNB (to determine total binding) or ligand plus 1 μM N-methyl atropine (to determine non-specific binding) for four hours at room temperature. The immunoprecipitates were centrifuged at 12 000 x g for 10 minutes at 4 °C, washed and assayed for levels of [^{3}H] ligand binding by liquid scintillation counting. The non-specific binding was subtracted from the total binding to yield specific binding (Figure 4.8).

Figure 4.8



M_3 receptor co-immunoprecipitation with PLD is affected by agonist stimulation.

The immunoprecipitates of HA-PLD1 or HA-PLD2 under control (basal) conditions and with 10 min stimulation by 100 μM of the M_3 receptor agonist carbachol (CCh) were assayed for the presence of M_3 receptor by [^{3}H]N-MeQNB binding. The non-specific binding (black bars) was subtracted from the total binding (grey bars) for each condition to yield specific binding. The non-immune IgG controls are shown for each condition. The M_3 receptor was specifically co-immunoprecipitated by both PLD1 and PLD2 under basal conditions. Upon stimulation by CCh, the association of M_3 with PLD1 was significantly attenuated (*$p<0.05$; Wilcoxon test, n=5). The co-immunoprecipitation of M_3 with PLD2 was not significantly reduced.

The results show that the immunoprecipitation of both HA-PLD1 and HA-PLD2 specifically pulled down the M_3 muscarinic receptor. Furthermore, the stimulation of the M_3 receptor by carbachol resulted in a significant reduction of the association of the M_3 receptor with PLD1 ($p<0.05$, Unpaired student t-test; n=5). The stimulation of the receptor also resulted in a modest decrease of the association with PLD2, however this was not statistically significant ($p>0.05$, Unpaired student t-test; n=5). These results support the findings of the M_3 receptor-directed immunoprecipitation of the PLD1 and PLD2 isozymes under basal conditions. Furthermore, the sensitivity of the radiolabelled assay compared to Western blot analysis indicates that there may have been a slight undetected decrease in association of the PLD2 isozyme with the M_3 receptor upon carbachol stimulation for 10 minutes (as was found for PLD1). These results therefore suggest that PLD1 and perhaps also PLD2 could potentially mediate any PLD-dependent facilitation of internalisation of the M_3 muscarinic receptor.

Visualising colocalisation of the M_3 receptor and PLD1 within COS 7 cells

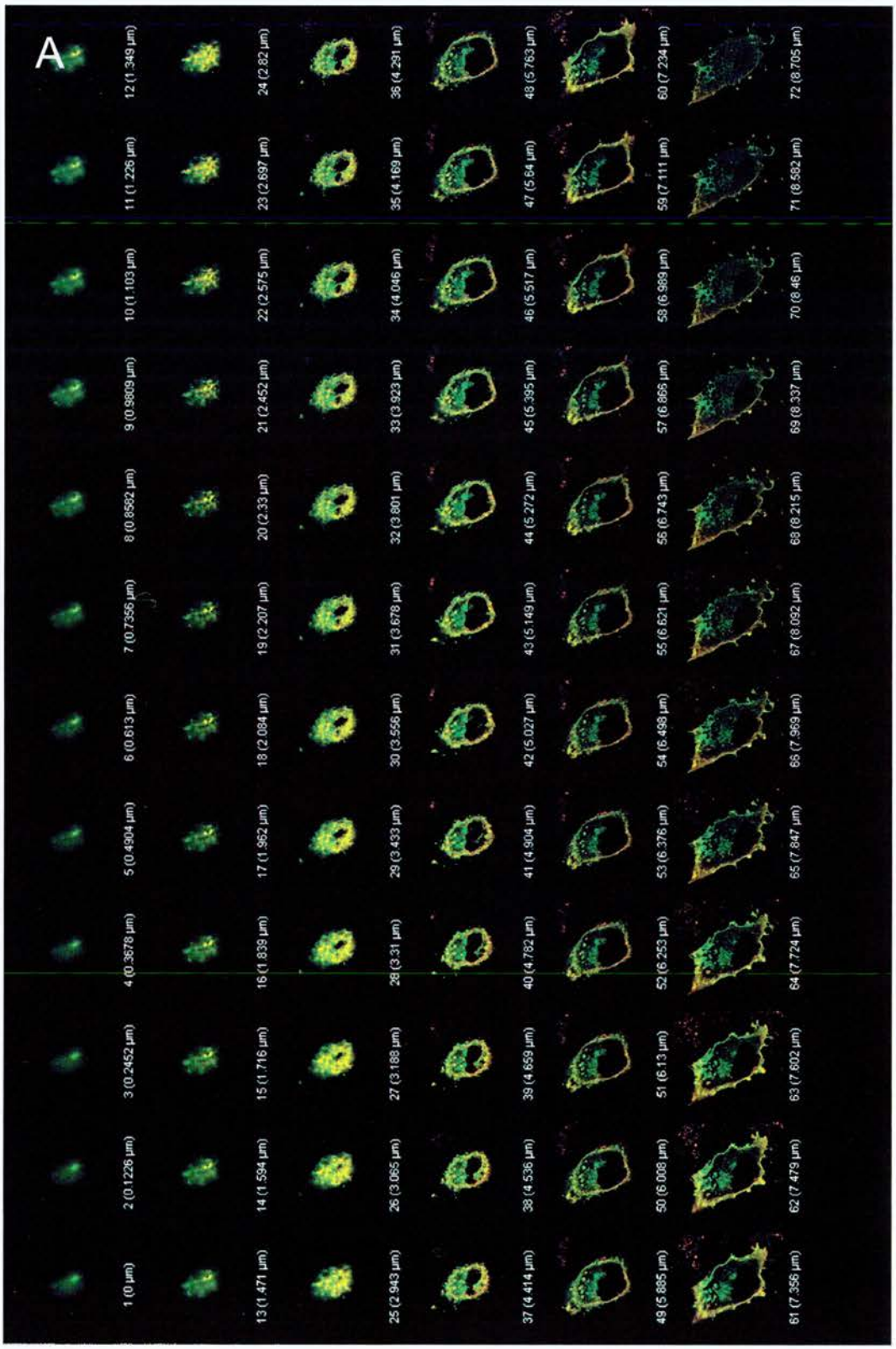
COS 7 cells, grown on glass coverslips, were transiently transfected with sFM_3 receptor and HA-PLD1 in a 1:1 ratio. The cells were serum-deprived for six hours before fixing, permeabilised and fixed overnight, and stained with primary mouse monoclonal anti-HA antibody clone 12CA5 (Roche Diagnostics) and rabbit polyclonal anti-M_3 antibody (kindly gifted by Dr A. Tobin), followed by Alexa Fluor goat anti-mouse (488 nm) and anti-rabbit (567 nm) secondary antibodies (Molecular Probes, Leiden, The Netherlands). Healthy cells that had been co-transfected were chosen at random then imaged by confocal microscopy, acquiring data at Nyquist sampling rates.

The cells imaged under basal conditions displayed a distribution of PLD1 (green) localised to the perinuclear regions, punctate intracellular structures and the plasma membrane, that was similar to that found previously. The M_3 receptor (red) was localised

primarily to the plasma membrane as expected (Figure 4.9). From empirical observations the area of potential colocalisation of the M_3 receptor with PLD appeared to be mainly at the plasma membrane.

Equivalent samples of co-transfected COS 7 cells, containing sFM_3 and HA-PLD1 as before, were stimulated with 100 μM of the muscarinic agonist carbachol for 10 minutes prior to fixing. These cells were imaged as for unstimulated cells (Figure 4.10). Agonist stimulation of the muscarinic receptor lead to an increase in recruitment of PLD1 to the plasma membrane in COS 7 cells, consistent with other reports of PLD1 translocating to the plasma membrane upon cellular stimulation (Morgan *et al.*, 1997; Brown *et al.*, 1998; Du *et al.*, 2003; Mitchell *et al.*, 2003). As well as the translocation of PLD1, the stimulated cells showed a marked internalisation of the M_3 receptor after 10 minutes of agonist stimulation (demonstrated with the large levels of red staining on intracellular structures). This yields additional information regarding the time course of the M_3 receptor internalisation. It has been shown that many GPCRs are desensitised within minutes and are endocytosed following this step, with some GPCRs held in a subcellular pool so that they can be re-inserted into the plasma membrane following agonist treatment (receptor recycling) and others being directed into a more degradative pathway (Zhang *et al.*, 1996; von Zastrow, 2003; Du *et al.*, 2004; Koch *et al.*, 2004). We have demonstrated that the M_3 receptor can be internalised within 10 minutes of agonist stimulation (Johnson *et al.*, 2004) and the confocal images support this finding. In addition, the distribution of the M_3 receptor in a compartment just below the plasma membrane may be consistent with the notion that receptors are present on early endosomes before being sorted for recycling or degradation by lysosomes (von Zastrow, 2003). PLD2 has been proposed to play a functional role in the resensitisation of the μ-opioid receptor back to the plasma membrane (Koch *et al.*, 2004), and so by analogy, the presence of the PLD1 at the plasma membrane here may potentially facilitate the re-insertion of the M_3 receptor back to the plasma membrane.

Figure 4.9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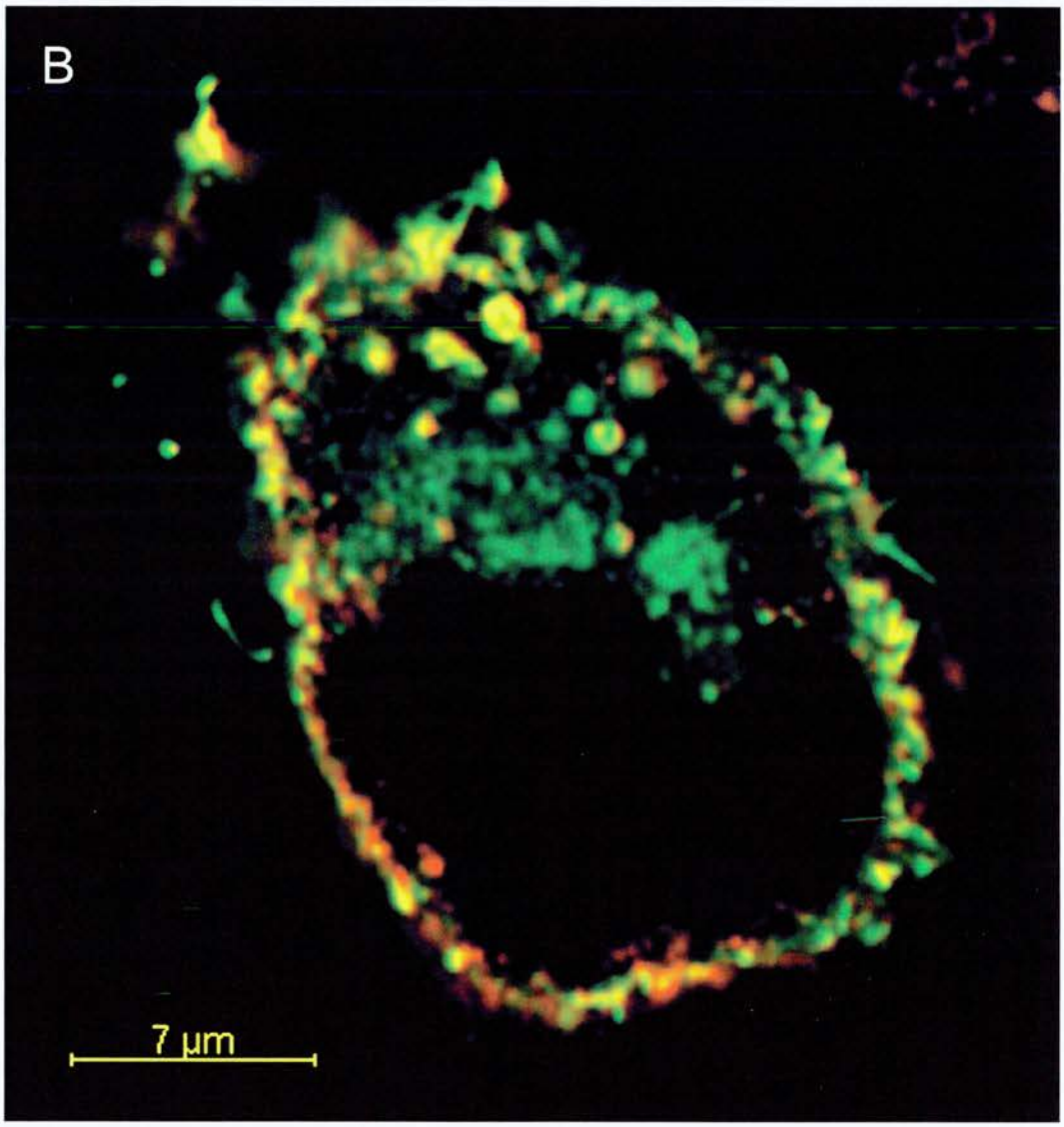
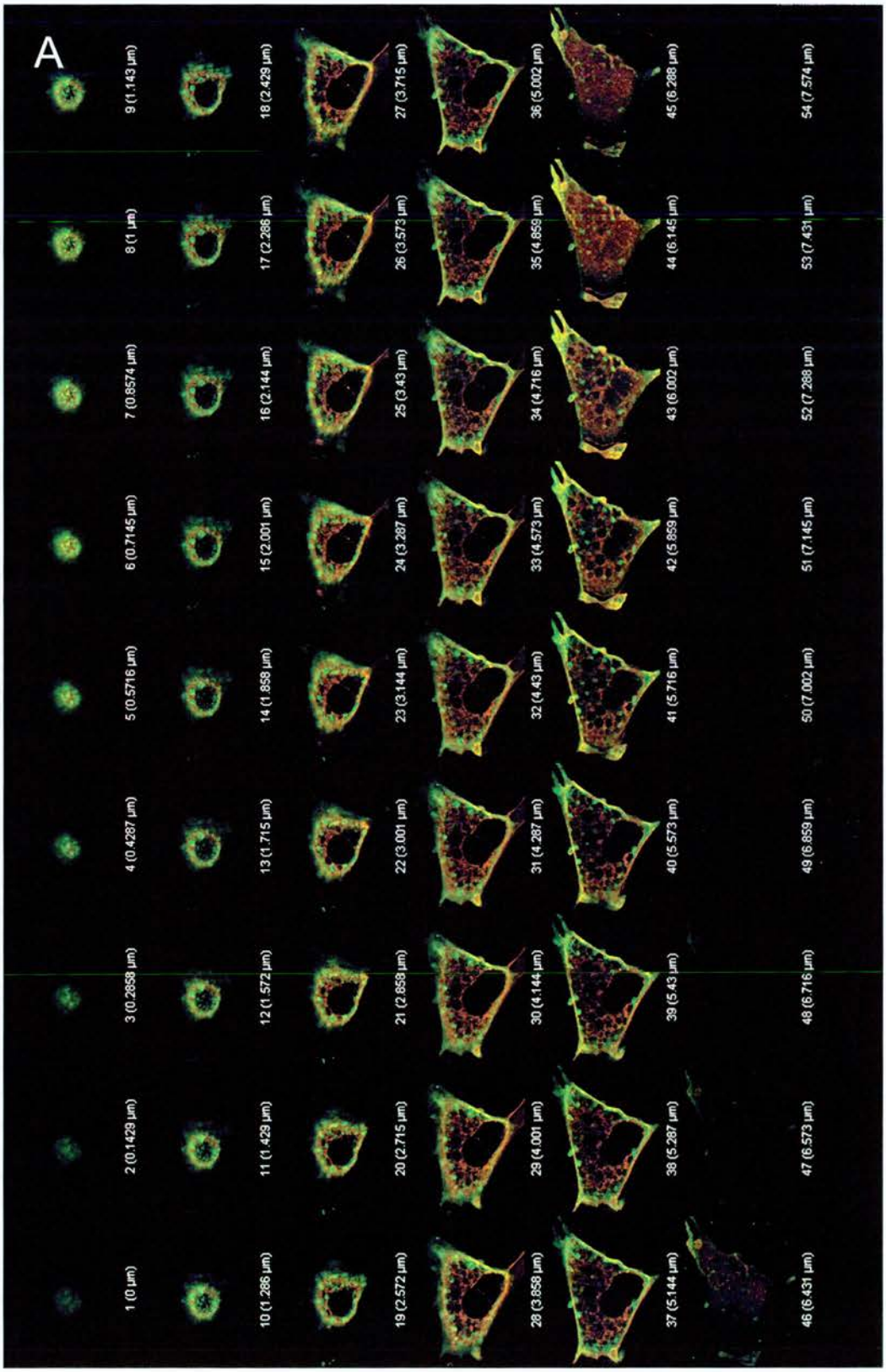


The localisation of the M_3 receptor and PLD1 in unstimulated COS 7 cells.

The gallery (A) and mid-section (B) views of an unstimulated COS 7 cell transiently transfected with sFM_3 receptor (red) and HA-PLD1 (green). The perinuclear distribution of PLD1 is indicative of PLD1 localising with the Golgi apparatus and can be seen clearly in the mid section view.

Figure 4.10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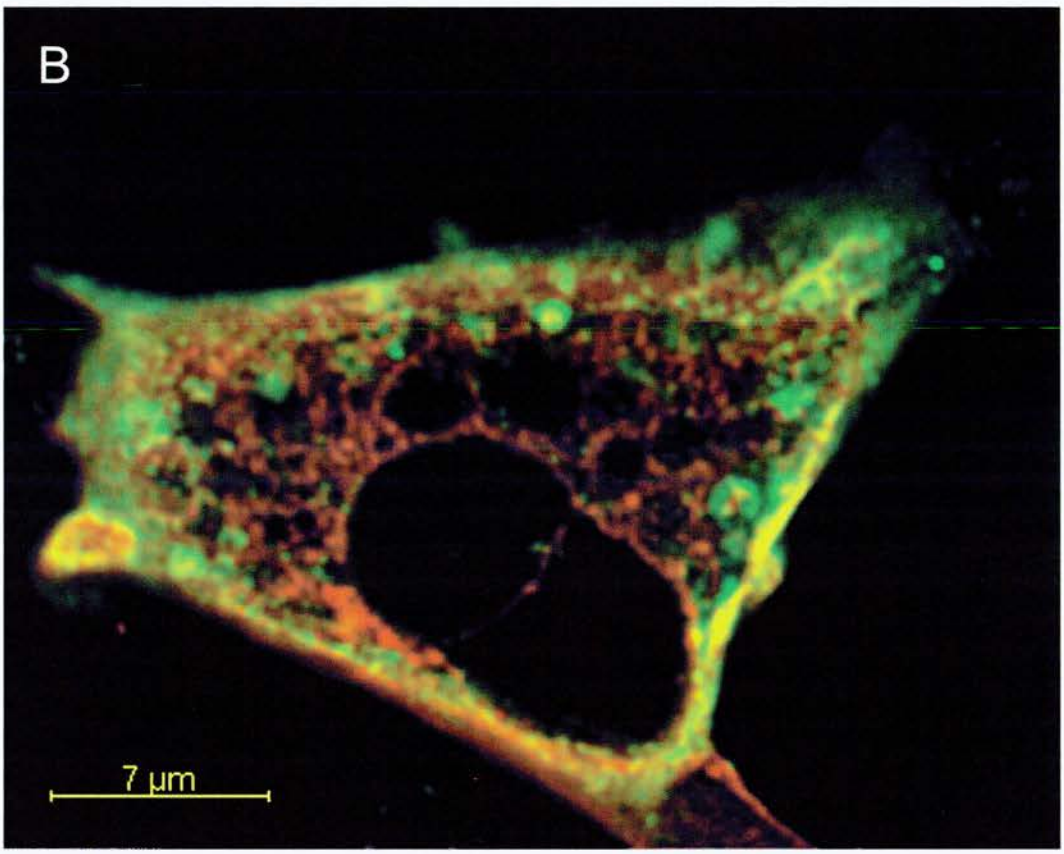

The localisation of the M_3 receptor and PLD1 in M_3 agonist stimulated COS 7 cells.

The gallery (A) and mid-section (B) views of an agonist stimulated COS 7 cell, transiently transfected with sFM_3 receptor (red) and HA-PLD1 (green). The M_3 agonist carbachol (100 µM for 10 minutes) was used to stimulate the cells. The translocation of PLD1 to the plasma membrane is apparent and there is also substantial internalisation of the M_3 receptor.

To examine the nature of the potential M_3 receptor:PLD interaction further, voxel colocalisation analysis studies were carried out on imaged resting and stimulated COS 7 cells containing M_3 and PLD1. Colocalisation describes the presence of two fluorochromes at the same (resolvable) physical location and is determined by voxels (three dimensional pixels) that contain two or more image channel intensities. This colocalisation can be expressed as a coefficient of correlation when all the voxels of one channel are plotted against all the voxels of another channel (2D voxel plot). A value of 1 indicates that all voxels within the dataset contain equal channel intensities (a perfect linear correlation) and is visualised on the voxel plot as a straight line with an equal rise and run passing through the origin. A value of 0 is indicative of no correlation between channel intensities and a value of –1 indicates inverse correlation. The 2D voxel plots for sFM_3 and HA-PLD1 in two unstimulated COS 7 cells (Figure 4.11A) and two carbachol stimulated COS 7 cells (Figure 4.11B) are shown. These plots indicate qualitatively that the colocalisation of M_3 and PLD1 is reduced with receptor stimulation. Colocalisation thresholds were maintained where possible, providing that channel bleed through was eliminated. Voxels above threshold intensity levels containing both red (sFM_3 receptor) and green (HA-PLD1) channel information were analysed for the correlation coefficient of colocalisation. Analysis revealed that the average colocalisation correlation coefficient for M_3 and PLD1 in resting cells was 0.48 ± 0.07 (n=6) and following M_3 receptor agonist stimulation (with 100 μM carbachol for 10 minutes) was 0.30 ± 0.10 (n=4) - a reduction of 38%. However this was not quite significant (p=0.07, Unpaired student t-test) (Figure 4.12A). Moreover, approximately $10.46 \pm 2.16\%$ of the total number of voxels above threshold levels were colocalised for sFM_3 and HA-PLD1 under basal conditions and this figure was reduced to $6.50 \pm 1.47\%$ upon agonist stimulation, indicating a reduction of colocalised voxels of 3.94% in the total dataset and a relative reduction of 38% (Figure 4.12B). Although colocalisation itself does not imply interaction, these data support the biochemical analysis and suggest that the

Figure 4.11

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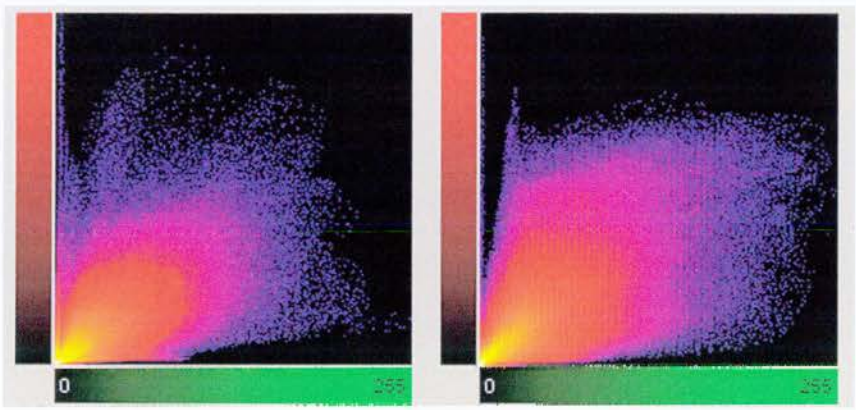

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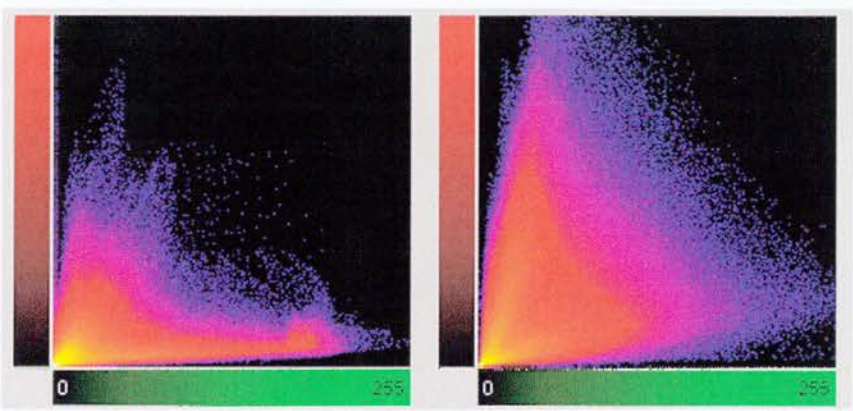
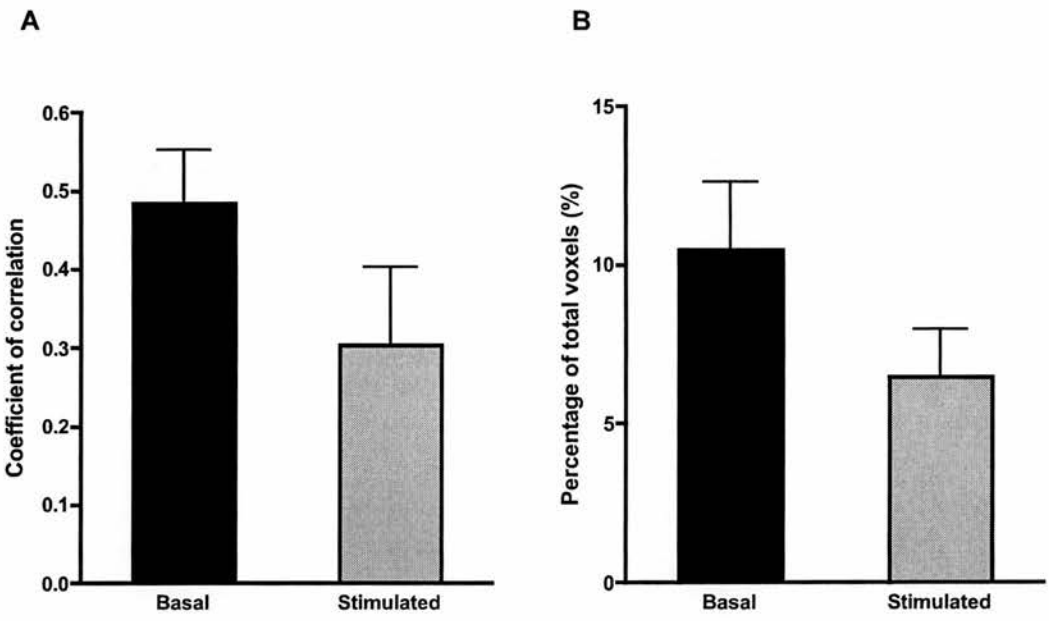


The colocalisation of the M_3 receptor and PLD1 is qualitatively affected by carbachol.

The 2-dimensional plots of all voxels from datasets containing channel intensities for the M_3 muscarinic receptor (red) and PLD1 (green) in basal COS 7 cells (A) and carbachol stimulated COS 7 cells (100 μM for 10 min) (B). A perfect correlation (where all voxels of one channel are colocalised with all voxels of another channel) would be a straight line of equal rise and run passing through the origin (with a gradient of 1). The colocalisation plots from the basal COS 7 cells (A) show a better correlation (and thus colocalisation) than those from agonist stimulated COS 7 cells (B).

Figure 4.12



The effect of agonist stimulation on the colocalisation of the M_3 receptor and PLD1.

The coefficient of correlation (indicating colocalisation) of voxels containing information for both channel intensities (M_3 and PLD1) for both resting COS 7 cells (n=6) and COS 7 cells that had been stimulated for 10 min with 100 μM of the M_3 agonist carbachol (n=4) is shown (A). A perfect correlation of colocalisation would have a value of 1, whereas no correlation would have a value of 0. The reduction is not quite significant (p=0.07, Unpaired student t-test). The percentage of voxels above thresholds that contained two channel intensities was measured as a proportion of the total voxels in the dataset and was plotted for both resting and stimulated COS 7 cells (B). The stimulation of the muscarinic receptor induces an apparent decrease in the number of voxels that contain both M_3 and PLD1 channel information and this implies a reduction in co-localisation.

interaction of the M_3 muscarinic receptor with PLD1 may be primarily localised at the plasma membrane in COS 7 cells and that agonist stimulation of the receptor can induce transient dissociation of the putative M_3 receptor/PLD1 complex.

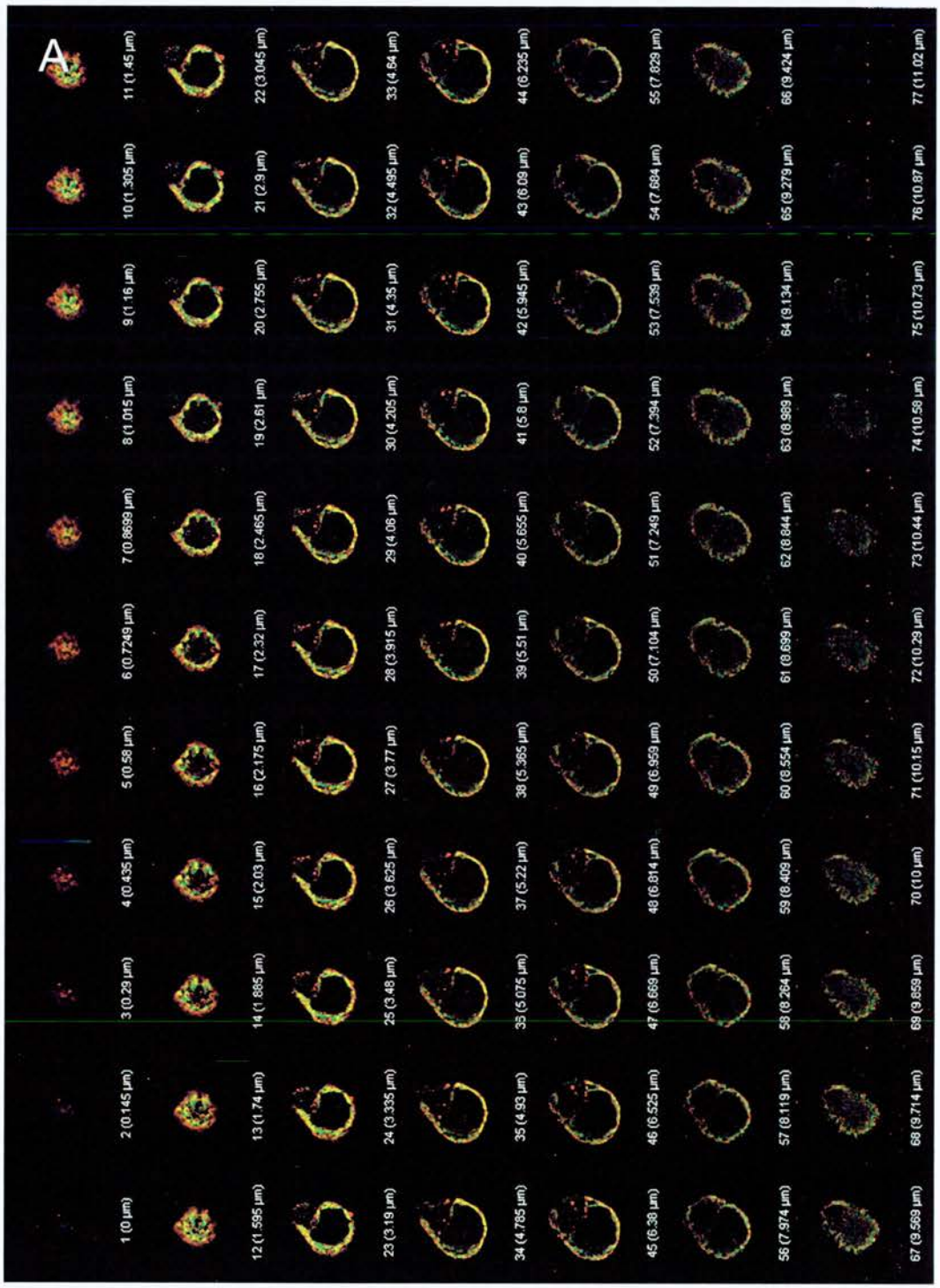
Visualising colocalisation of the M_3 receptor and PLD2 within COS 7 cells

Imaging studies were repeated in COS 7 cells transiently transfected with sFM_3 receptor and HA-PLD2 cDNAs in a 1:1 ratio. The cells were grown on coverslips, serum deprived for six hours and stimulated where necessary with 100 μM carbachol for 10 minutes. The cells were permeabilised and fixed overnight, and stained with primary mouse monoclonal anti-HA antibody clone 12CA5 (Roche Diagnostics) and rabbit polyclonal anti-M_3 antibody (kindly gifted by Dr A. Tobin), followed by Alexa Fluor goat anti-mouse (488 nm) and anti-rabbit (567 nm) secondary antibodies (Molecular Probes). The cells were then imaged by confocal microscopy as before.

In unstimulated cells, the M_3 receptor (red) localised mainly to the plasma membrane. The majority of the PLD2 isozyme (green) also associated closely with the plasma membrane as found previously (Figure 4.13). There was no detectable level of PLD2 in the Golgi or nucleus of the cells - contrasting with the report from Freyberg and colleagues (Freyberg *et al.*, 2002) but actively consistent with most other relevant studies.

In agonist-stimulated cells, the M_3 receptor appeared to internalise and was present in discrete intracellular structures. The PLD2 remained mainly associated with the plasma membrane, with a small proportion diffusely localised under the plasma membrane (Figure 4.14). The PLD2 appeared to form areas of high concentrations around the plasma membrane that could potentially be consistent with localisation in caveolae (Czarny *et al.*, 1999).

Figure 4.13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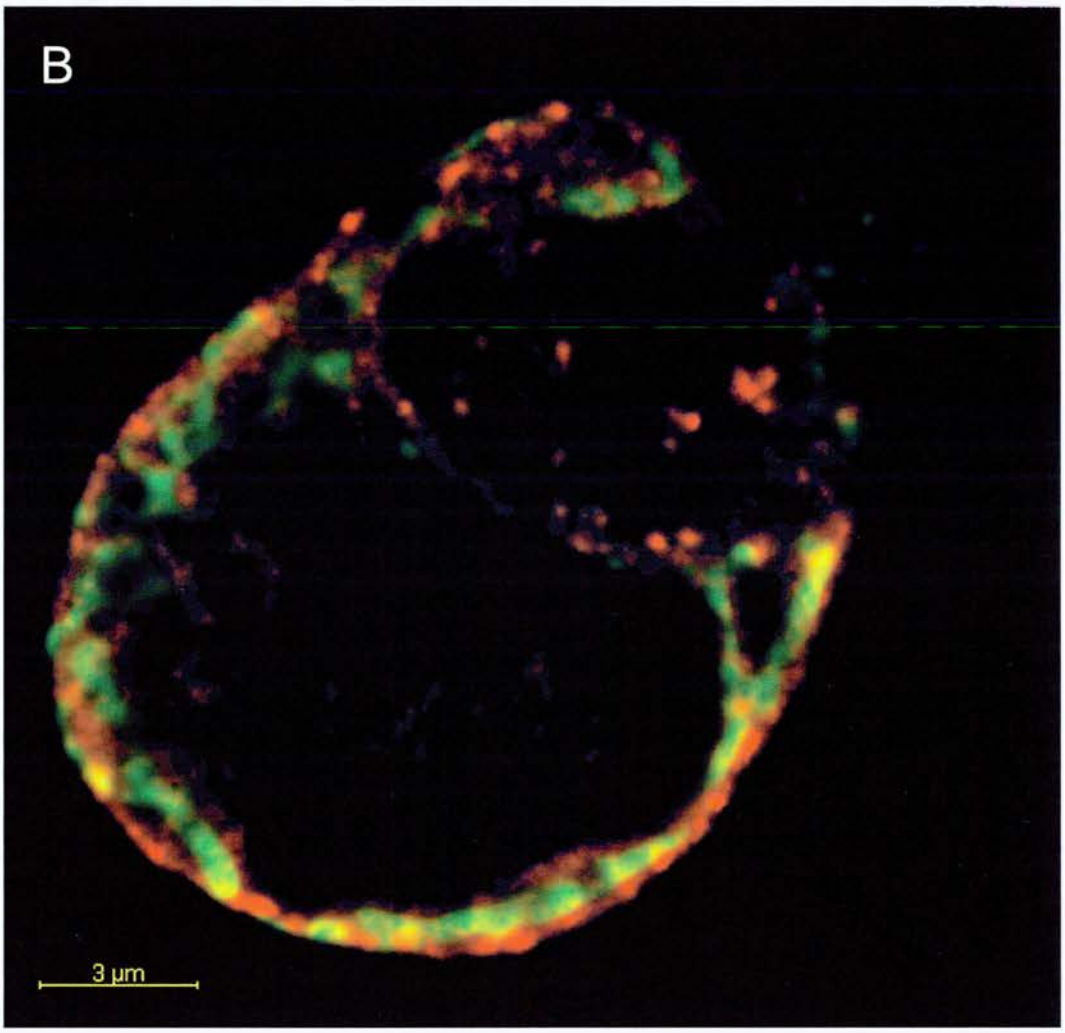
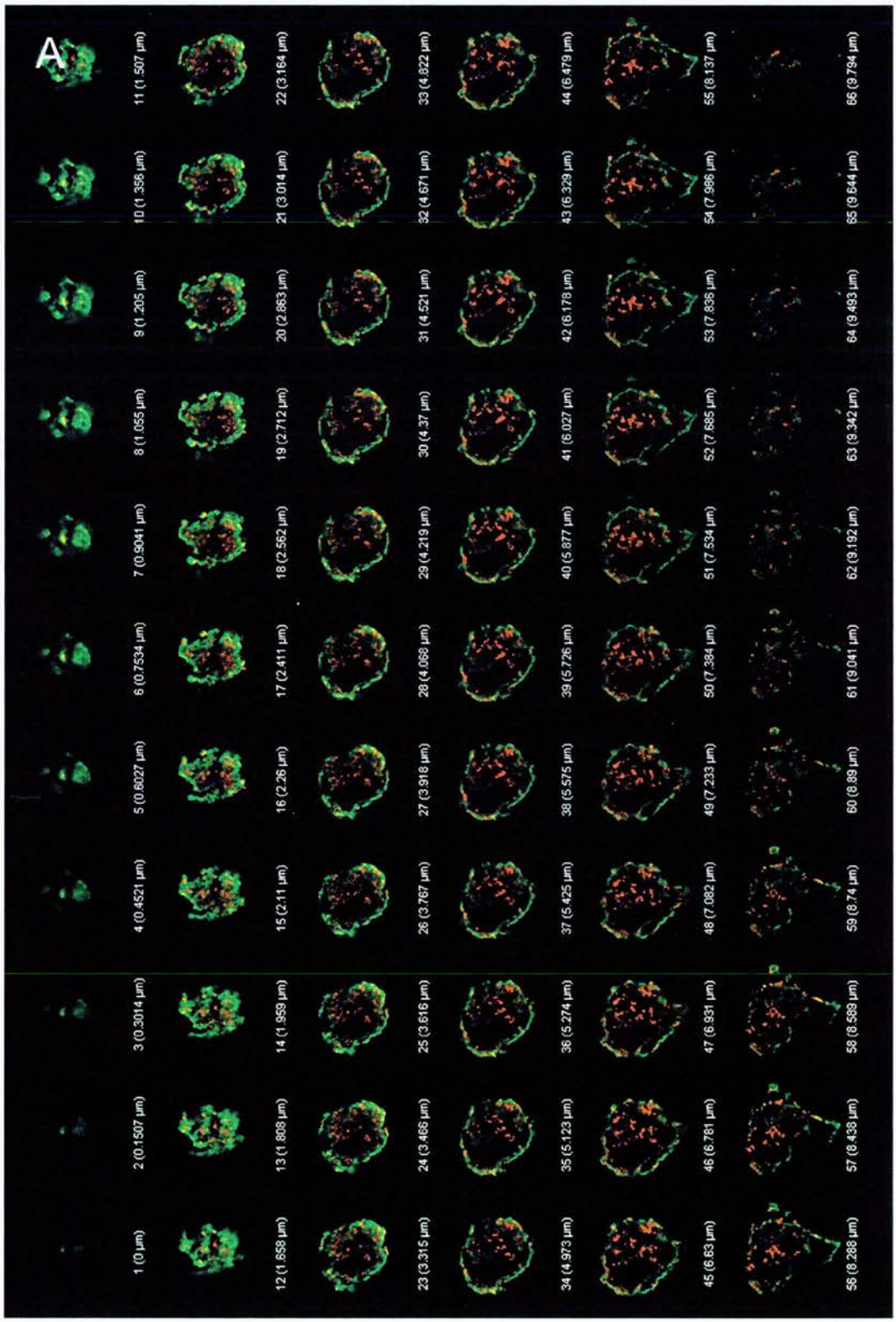


The localisation of the M_3 receptor and PLD2 in unstimulated COS 7 cells.

The gallery (A) and mid-section (B) views of an unstimulated COS 7 cell transiently transfected with sFM_3 receptor (red) and HA-PLD2 (green). The M_3 receptor and PLD2 are localised at the plasma membrane of the cell.

Figure 4.14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25 (3.616 µm)
26 (3.767 µm)
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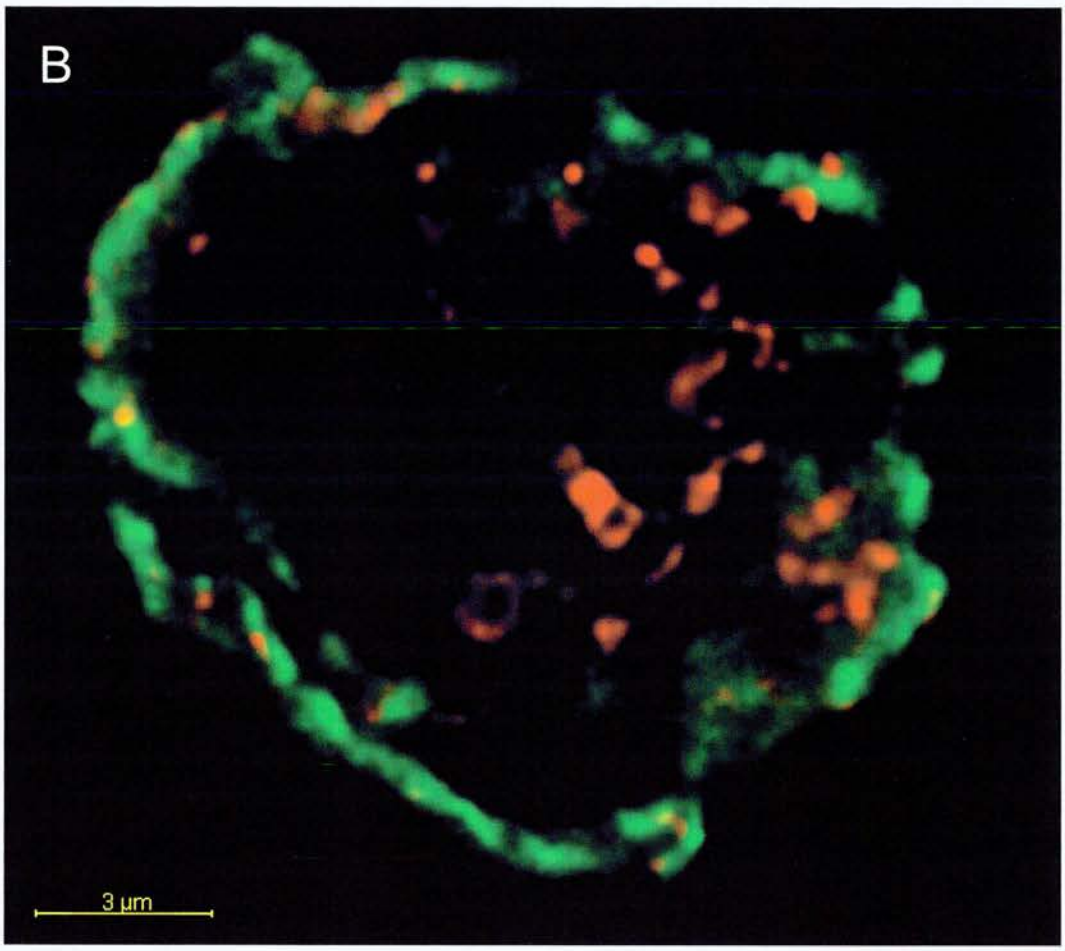
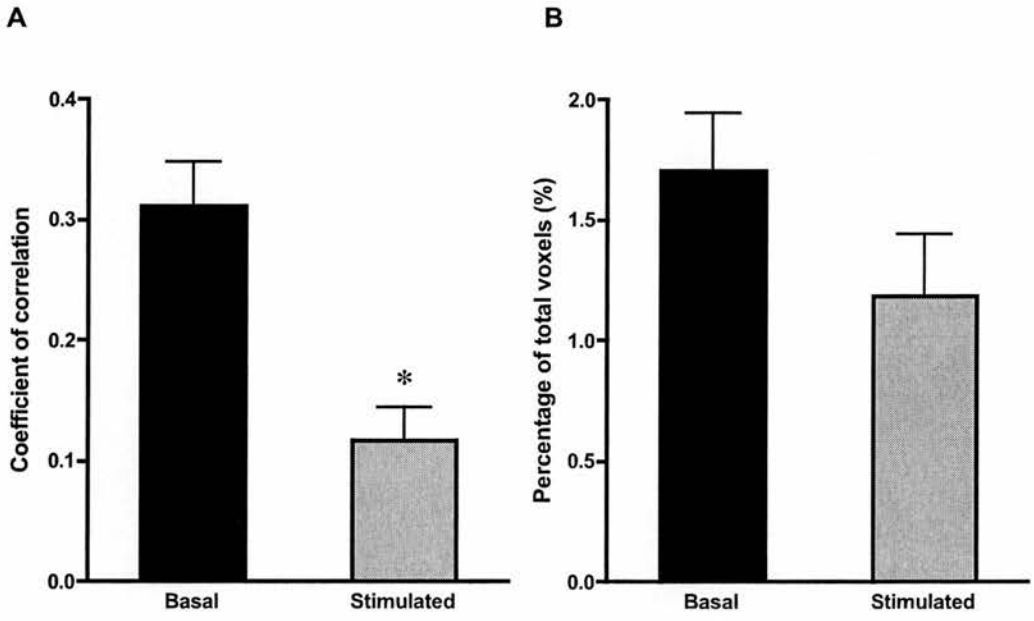


The localisation of the M_3 receptor and PLD2 in M_3 agonist stimulated COS 7 cells.

The gallery (A) and mid-section (B) views of an agonist stimulated COS 7 cell, transiently transfected with sFM_3 receptor (red) and HA-PLD2 (green). The M_3 agonist carbachol (100 µM for 10 minutes) was used to stimulate the cells.

The correlation coefficent of colocalisation was determined for both unstimulated and stimulated cells as before (Figure 4.15). In unstimulated cells, the mean colocalisation correlation coefficient was determined to be 0.31 ± 0.04 (n=6) and in stimulated cells, this was significantly reduced to 0.12 ± 0.03 (n=6) ($p<0.05$, Unpaired student t-test), a relative reduction of 61%. The value under basal conditions was surprising, as PLD2 is more associated with the plasma membrane than PLD1, yet the colocalisation coefficient under basal conditions is lower for the colocalisation of PLD2 and M_3 than it is for PLD1 and M_3. It may be the case that although the PLD2 and M_3 are localised to the same cellular compartment, the precise incidence of colocalisation may be lower due to PLD2 being in a different membrane microdomain than the receptor under physiological conditions. The value for potential colocalisation does not make any implications about whether the proteins interact, but only whether they occupy the same space. This may mean that proteins can colocalise without physically interacting, or alternatively that the localisation of the M_3 receptor and PLD at any time does not necessarily reflect the population of these molecules that are physically interacting, as assessed by co-immunoprecipitation. The average number of colocalised voxels was also determined for the M_3 receptor and PLD2; in unstimulated cells the average was 1.70 ± 0.29% (n=6) and this reduced to 1.18 ± 0.26% (n=6) in stimulated cells, a relative reduction of 31%.

Figure 4.15



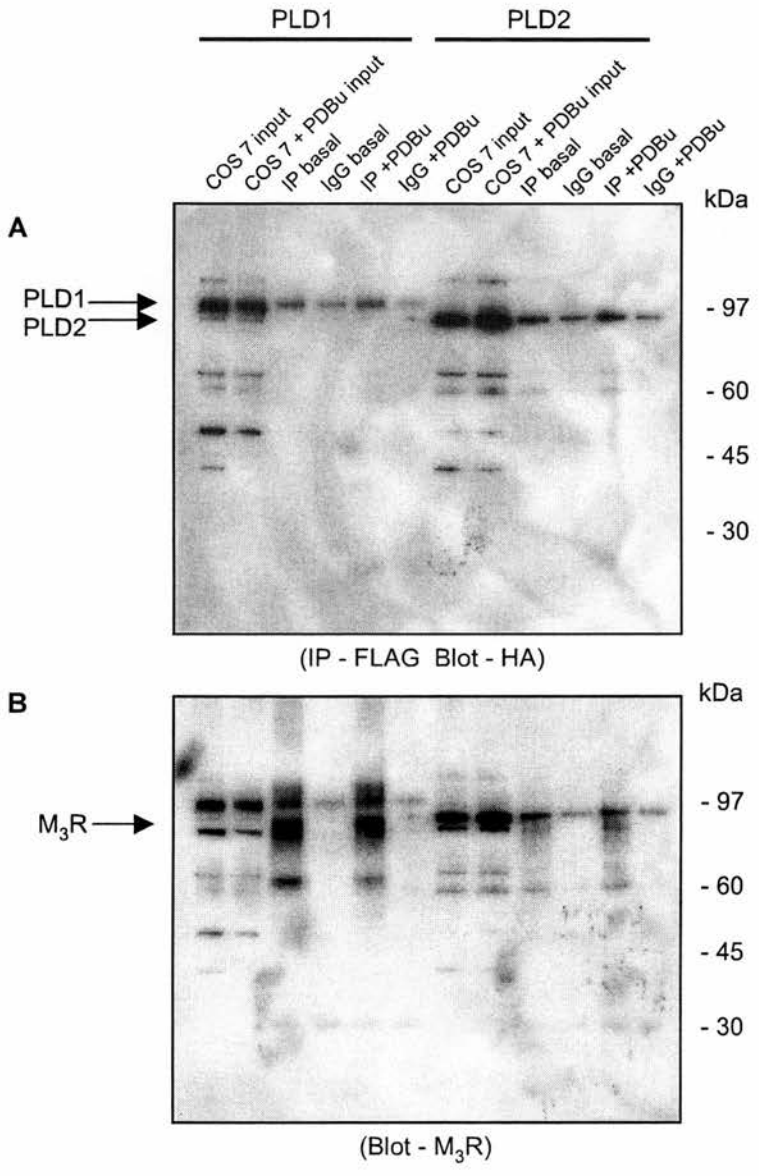
The effect of agonist stimulation on the colocalisation of the M_3 receptor and PLD2.

The coefficient of correlation of voxels containing information for both channel intensities (M_3 and PLD2) for both resting COS 7 cells (n=6) and COS 7 cells that had been stimulated for 10 min with 100 μM of the M_3 agonist carbachol (n=6) is shown (A). The stimulation by carbachol results in a significant decrease in the colocalisation of PLD2 with the M_3 receptor (*$p<0.05$; Unpaired student t-test). The percentage of voxels above thresholds that contained two channel intensities was measured as a proportion of the total voxels in the dataset and was plotted for both resting and stimulated COS 7 cells (B). The stimulation of the muscarinic receptor induces an apparent decrease in the number of voxels that contain both M_3 and PLD2 channel information similarly but smaller than that for the colocalisation of M_3 and PLD1.

The effect of PKC stimulation on the association of the M_3 receptor and PLD

To determine whether the change in association of M_3 and PLD was due to the activation state of PLD, the co-immunoprecipitation of PLD1 and PLD2 with the M_3 receptor was repeated with the stimulation of PLD by an alternative receptor-independent route, by using phorbol 12,13-dibutyrate (PDBu) to stimulate the PLD activator, protein kinase C (PKC). COS 7 cells were transiently transfected with the sFM_3 receptor and HA-PLD1 or HA-PLD2. The cells were stimulated where necessary with 500 nM PDBu for 10 minutes to activate PKC and subsequently PLD. The FLAG tag of the M_3 receptor was immunoprecipitated by 3 μg/ml mouse anti-FLAG antibody clone M2 (Sigma Aldrich) or 3 μg/ml non-immune mouse IgG as a control, followed by 40 μl/ml of a 1:1 suspension of Protein-G Sepharose in immunoprecipitation buffer. The beads were washed and co-immunoprecipitates lysed from the matrix, separated by SDS-PAGE, Western blotted and visualised using rat anti-HA HRP-conjugated primary antibody (Roche Diagnostics) (Figure 4.16). The co-immunoprecipitation of PLD1 and PLD2 was not discernibly affected by PDBu stimulation in COS 7 cells. This suggests that it may not be the activation state of PLD itself that causes the dissociation from the M_3 receptor. However, the activation of PKC by phorbol ester may have additional complicating effects on either the receptor or PLD, or on the mechanism of their intracellular trafficking.

Figure 4.16



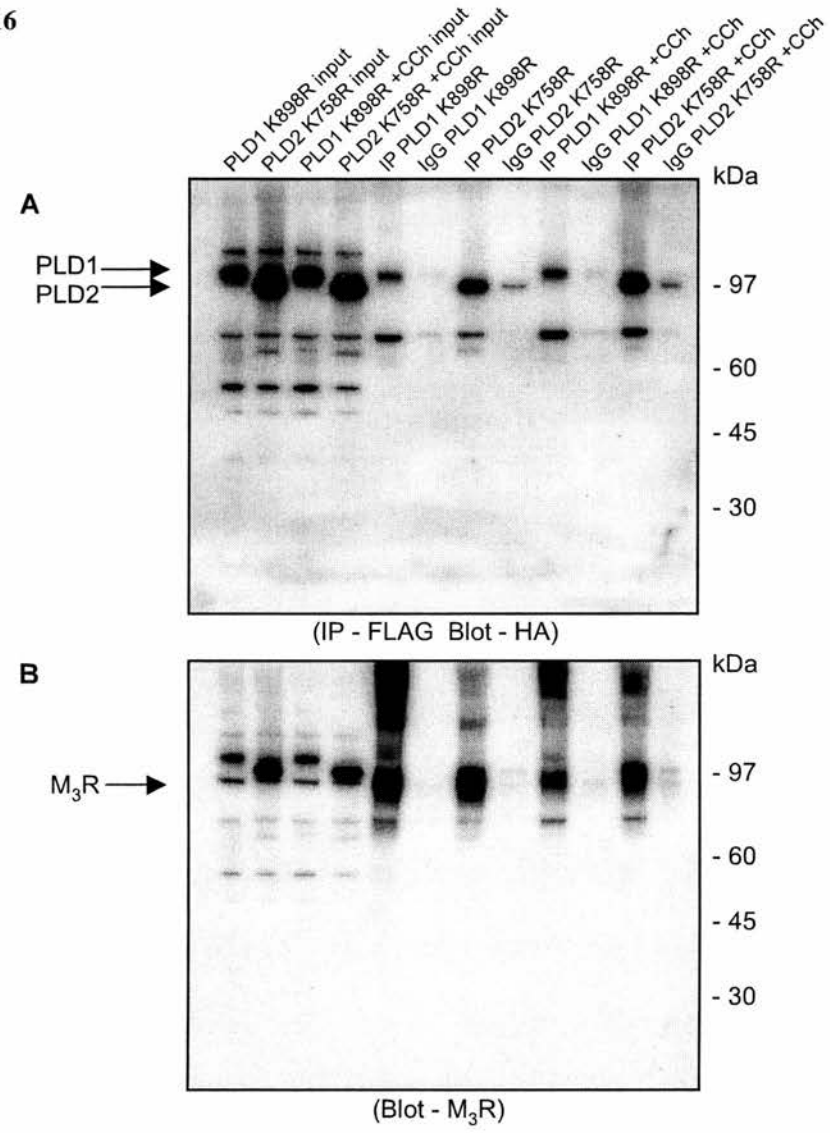
PLD association with the M_3 receptor is not affected by PKC stimulation.

Wild type HA-PLD1 or HA-PLD2 were transiently transfected with the sFM_3 receptor into COS 7 cells. The cells were stimulated with 500 nM PDBu for 10 min where indicated and immunoprecipitates directed against the FLAG tag of the receptor were pulled down. The co-immunoprecipitating levels of the PLDs, visualised with anti-HA, are shown (A). The stimulation by PBDu does not cause a reduction in associating PLD levels compared to basal controls. The levels of immunoprecipitates are also shown (B).

The effect of catalytically inactivating mutations in PLD1/2 on their association with the M_3 receptor

The HA-tagged catalytically inactive PLD mutants, HA-K898R PLD1 and HA-K758R PLD2 (kindly gifted by Dr M. Frohman), were transiently co-transfected into COS 7 cells with the sFM_3 receptor cDNAs in a 1:1 ratio. The cells were serum-deprived for 16 hours and were stimulated where necessary with 100 μM of the M_3 receptor agonist carbachol for 10 minutes. The levels of catalytically inactive PLDs co-immunoprecipitating with the M_3 receptor, visualised with anti-HA (Roche Diagnostics), are shown in Figure 4.17. Specific association of the dominant negative PLD1 and PLD2 with the M_3 receptor was observed under basal conditions, as previously shown with the wild type PLD isozymes. Upon M_3 stimulation by carbachol, the levels of co-immunoprecipitating K898R PLD1 and K758R PLD2 were not affected compared to basal levels. This contrasts with the findings made with the wild type PLD1, which was transiently reduced with stimulation of the M_3 receptor by carbachol. This implies that the catalytic activity of PLD1 may be necessary for the dissociation from the M_3 receptor, whereas PLD1 activation by PKC rather than the M_3 receptor is not sufficient to cause dissociation. This may imply that changes in the receptor as well as PLD activation are necessary to allow dissociation. Furthermore, we have demonstrated that the catalytic activity of both PLD1 and PLD2 is necessary for facilitation of M_3 receptor internalisation as the presence of K898R PLD1 and K758R PLD2, as well as the PLD inhibitor calphostin C, are inhibitory to the agonist-induced endocytosis of the M_3 muscarinic receptor from the cell surface (Johnson *et al.*, 2004).

Figure 4.16



Mutant PLD association with the M_3 receptor is not agonist dependent.

The catalytically inactive PLD mutants, HA-PLD1 K989R and HA-PLD2 K758R, were transiently transfected with the sFM_3 receptor into COS 7 cells. The cells were stimulated with 100 μM carbachol (CCh) for 10 min where indicated and immunoprecipitates directed against the FLAG tag of the receptor were pulled down. The co-immunoprecipitating levels of the dominant negative PLDs, visualised with anti-HA, are shown (A). The stimulation by carbachol, does not cause a reduction in associating PLD levels compared to basal controls. The levels of immunoprecipitates are also shown (B).

Summary

The findings in this chapter demonstrate that PLD1 and PLD2 have an intracellular distribution in COS 7 cells that is in broad agreement with other reports. The PLD isozymes can both interact with the M_3 muscarinic receptor under basal conditions (shown by co-immunoprecipitation studies) and this interaction is probably at a site provided by regions of both the third intracellular loop and the carboxy-terminal tail domain of the receptor (shown by GST-fusion studies). Stimulation of the M_3 receptor by the agonist carbachol leads to a significant but transient decrease in the co-immunoprecipitation of the M_3 receptor with wild type PLD1 but not wild type PLD2 at ten minutes. Dissociation of the M_3 receptor and PLD1 is transient and is rapidly restored to basal levels by twenty minutes. The dissociation of PLD and the M_3 receptor was not apparent with the catalytically inactive mutants of either PLD isozyme, nor could it be caused by PKC-mediated stimulation of the wild type PLDs. Furthermore, confocal microscopy revealed that the colocalisation of either PLD1 or PLD2 with the M_3 receptor was reduced by agonist stimulation and this may support the biochemical observations. However, more experimental data would be necessary to further elucidate the mechanisms involved. The use of real-time cell imaging of cells expressing fluorescently tagged receptor and PLD isozyme constructs would be an elegant method of delineating the changing mechanisms involved in the receptor-PLD interaction upon cellular stimulation. The use of fluorescence resonant energy transfer (FRET), whereby the close association of proteins can be determined from the transfer of energy from one fluorophore on one protein to another on a separate protein (and thus indicating interaction) would also be extremely useful in this kind of cellular system. Additionally it would be desirable to use high affinity antibodies for the native M_3 receptor and PLD isozymes, as this would eliminate any inconsistencies that may arise when looking at a transiently transfected population of cells.

These findings are consistent with the hypothesis that the association of PLD isozymes (PLD1 or PLD2) with the M_3 receptor may make a functional contribution to agonist-induced desensitisation, endocytosis (sequestration) and perhaps resensitisation of the M_3 receptor as proposed by other groups working with other GPCRs (Koch *et al.*, 2003; Du *et al.*, 2004; Koch *et al.*, 2004). One mechanism for which PLD activity has been demonstrated to be important is in the initial stages of the assembly of clathrin coated pits (Arneson *et al.*, 1999; Lee *et al.*, 2000). Phosphatidic acid, the product of PLD activity, is necessary as a cofactor for the efficient assembly of the AP-2 adapter complex on lysosomes (Arneson *et al.*, 1999) and AP-2 has been shown to be important for the endocytosis of the β-adrenergic receptor (Laporte *et al.*, 1999). Phosphatidic acid is thought to affect the physical properties of the lipid bilayer to allow efficient budding of the vesicle away from the plasma membrane (Andresen *et al.*, 2002). The results found in this study are consistent with the notion that activation of PLD may facilitate endocytic sequestration of the M_3 receptor away from the plasma membrane following agonist stimulation and desensitisation.

Chapter 5:

The interaction of 14-3-3 with PLD

Introduction

14-3-3 proteins have many intracellular binding partners (Aitken, 2002), among them are proteins involved in cell cycle regulation (Pallas *et al.*, 1994), proteins involved in apoptosis (Zha *et al.*, 1996; Masters and Fu, 2001) and proteins involved with signalling mechanisms (Toker *et al.*, 1992; Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Bonnefoy-Berard *et al.*, 1995; Benzing *et al.*, 2000). The ability to interact with many (but not all) of these binding partners depends upon the presence of the c-Raf-like RSxpSxP phosphoserine binding motif (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The interaction of 14-3-3 with enzymes such as phosphatidylinositol 3-kinase (PI3K) (Bonnefoy-Berard *et al.*, 1995) and the regulators of G-protein signalling (Benzing *et al.*, 2000) has implicated the importance of 14-3-3 in certain cellular signalling pathways. 14-3-3 may act to coordinate binding partners to facilitate interaction, sequester proteins to prevent interaction or target proteins to specific compartments for activation. Isoforms of 14-3-3 have been shown to be present at intracellular and plasma membranes in other studies (Celis *et al.*, 1988; Jones *et al.*, 1995b) and in this study it has already been shown that 14-3-3 is present in both the cytosol and at the plasma membrane.

Analysis of the human PLD1 isozyme sequence reveals that it contains a putative 14-3-3 binding site with the residues RSLSYP (712-717) (Figure 5.1A). Scansite analysis of this domain reveals that it is a high stringency 14-3-3 mode 1 phosphoserine recognition site (Obenauer *et al.*, 2003). Human PLD2 contains two similar threonine-based sequences, RLLTMS (172-177) or KTPTYP (573-579) that may also potentially be 14-3-3 recognition motifs (Figure 5.1B), however these domains are only recognised with a medium stringency threshold (Obenauer *et al.*, 2003). In this chapter, the interaction of 14-3-3 isoforms with the PLD isozymes is investigated, along with the subcellular localisation and colocalisation of the proteins. In addition, the functional implications of 14-3-3 expression *in vivo* are investigated using whole cell PLC and PLD signalling assays.

Figure 5.1

A

```
   1  MSLKNEPRVN TSALQKIAAD MSNIIENLDT RELHFEGEEV DYDVSPSDPK IQEVYIPFSA IYNTQGFKEP
  71  NIQTYLSGCP IKAQVLEVER FTSTTRVPSI NLYTIELTHG EFKWQVKRKF KHFQEFHREL LKYKAFIRIP
 141  IPTRRHTFRR QNVREEPREM PSLPRSSENM IREEQFLGRR KQLEDYLTKI LKMPMYRNYH ATTEFLDISQ
 211  LSFIHDLGPK GIEGMIMKRS GGHRIPGLNC CGQGRACTRW SKRWLIVKDS FLLYMKPDSG AIAFVLLVDK
 281  EFKIKVGKKE TETKYGIRID NLSRTLILKC NSYRHARWWG GAIEEFIQKH GTNFLKDHRF GSYAAIQENA
 351  LAKWYVNAKG YFEDVANAME EANEEIFITD WWLSPEIFLK RPVVEGNRWR LDCILKRKAQ QGVRIFIMLY
 421  KEVELALGIN SEYTKRTLMR LHPNIKVMRH PDHVSSTVYL WAHHEKLVII DQSVAFVGGI DLAYGRWDDN
 491  EHRLTDVGSV KRVTSGPSLG SLPPAAHESM ESLRLKDKNE PVQNLPIQKS IDDVDSKLKG IGKPRKFSKF
 561  SLYKQLHRHH LHDADSISSI DSTS***SYFNHY*** ***RSHHNLIHGL*** ***KPHFKLPHPS*** ***SESEQGLTRP*** ***HADT***GSIRSL
 631  QTGVGELHGE TRFWHGKDYC NFVFKDWVQL DKPFADFIDR YSTPRMPWHD IASAVHGKAA RDVARHFIQR
 701  WNFTKIMKSK **YRSLSYP**FLL PKSQTTAHEL RYQVPGSVHA NVQLLRSAAD WSAGIKYHEE SIHAAYVHVI
 771  ENSRHYIYIE NQFFISCADD KVVFNKIGDA IAQRILKAHR ENQKYRVYVV IPLLPGFEGD ISTGGGNALQ
 841  AIMHFNYRTM CRGENSILGQ LKAELGNQWI NTISFCGLRT HAELEGNLVT ELIYVHSKLL IADDNTVIIG
 911  SANINDRSML GKRDSEMAVI VQDTETVPSV MDGKEYQAGR FARGLRLQCF RVVLGYLDDP SEDIQDPVSD
 981  KFFKEVWVST AARNATIYDK VFRCLPNDEV HNLIQLRDFI NKPVLAKEDP IRAEEELKKI RGFLVQFPFY
1051  FLSEESLLPS VGTKEAIVPM EVWT
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B

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   1  MTATPESLFP TGDELDSSQL QMESDEVDTL KEGEDPADRM HPFLAIYELQ SLKVHPLVFA PGVPVTAQVV
  71  GTERYTSGSK VGTCTLYSVR LTHGDFSWTT KKKYRHFQEL HRDLLRHKVL MSLLPLARFA VAYSPARDAG
 141  NREMPSLPRA GPEGSTRHAA SKQKYLENYL N**RLLTMS**FYR NYHAMTEPLE VSQLSFIPDL GREGLEGMIR
 211  KRSGGHRVPG LTCCGRDQVC YRWSKRWLVV KDSFLLYMCL ETGAISFVQL FDPGFEVQVG KRSTEARHGV
 281  RIDTSHRSLI LKCSSYRQAR WWAQEITELA QGPGRDFLQL HRHDSYAPPR PGTLARWFVN GAGYFAAVAD
 351  AILRAQEEIF ITDWWLSPEV YLKRPAHSDD WRLDIMLKRK AEEGVRVSIL LFKEVELALG INSGYSKRAL
 421  MLLHPNIKVM RHPDQVTLWA HBEKLLVVDQ VVAFLGGLDL AYGRWDDLHY RLTDLGDSSE SAASQPFTPR
 491  PDSPATPDLS HNQFFWLGKD YSNLITKDWV QLDRPFEDFI DRETTPRMPW RDVGVVVHGL PARDLARHFI
 561  QRWNFTKTTK AKY**KTPTYP**V LLPKSTSTAN QLPFTLPGGQ CTTVQVLRSV DRWSAGTLEN SILNAYLHTI
 631  RESQHFLYIE NQFFISCSDG RTVLNKVGDE IVDRILKAHK QGWCYRVYVL LPLLPGFEGD ISTGGGNSIQ
 701  AILHFTYRTL CRGEYSILHR LKAAMGTAWR DYISICGLRT HGELGGHPVS ELIYIHSKVL IADDRTVIIG
 771  SANINDRSLL GKRDSELAVL IEDTETEPSL MNGAEYQAGR FALSLRKHCF GVILGANTRP DLDLRDPICD
 841  DFFQLWQDMA ESNANIYEQI FRCLPSNATR SLRTLREYVA VEPLATVSPP LARSELTQVQ GHLVHFPLKF
 911  LEDESLLPPL GSKEGMIPLE VWT
```

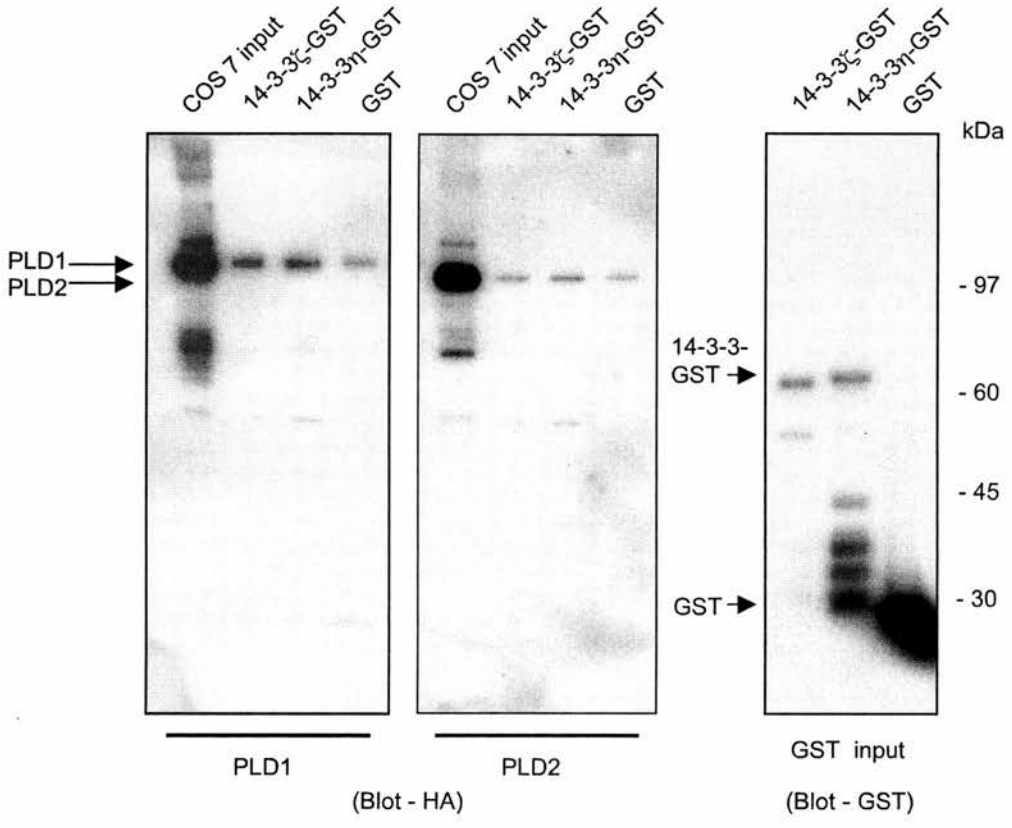
Sequences of human PLD1 and PLD2, showing the putative 14-3-3 recognition motifs.

The amino acid sequences of human phospholipase D1a (A) and human phospholipase D2 (B) are shown. The amino acid residues 712-717 in PLD1 are very similar to the c-Raf based 14-3-3 recognition motif RSxpSxP. The consensus sequences 172-177 or 573-579 in PLD2 may also be 14-3-3 recognition domains. These consensus sequences are underlined and in bold. The italicised region in PLD1a (585-623) is substituted for an asparagine (N) residue in the splice variant PLD1b (Hammond *et al.*, 1997).

14-3-3 isoforms interact with PLD1 and PLD2 in vitro

To determine whether PLD isozymes could interact with isoforms of 14-3-3, as predicted by the recognition sequence, glutathione S-transferase (GST) constructs of full length 14-3-3 isoforms (GST-14-3-3ζ and GST-14-3-3η) were immobilised onto glutathione Sepharose 4B beads. Cellular extracts from serum-deprived COS 7 cells, transiently transfected with HA-PLD1 and HA-PLD2 were incubated overnight with the prepared GST fusion protein beads. GST alone was used as a negative control. The bead-associated proteins were lysed, separated by SDS-PAGE, Western blotted and visualised using rat anti-HA HRP-conjugated primary antibody (Roche Diagnostics, East Sussex, UK). The Western blots of the bait associated proteins are shown in Figure 5.2 together with blots for the input levels of 14-3-3-GST fusion proteins. Both PLD1 and PLD2 specifically associated with both 14-3-3ζ and 14-3-3η above control levels in the *in vitro* GST-fusion protein binding assay. The binding of PLD1 to both the 14-3-3 constructs was consistently similar (1.89 ± 0.16 fold over GST control for 14-3-3ζ and 2.10 ± 0.20 fold over GST control for 14-3-3η, n=4). The binding of PLD2 appeared to have a higher affinity for the 14-3-3η isoform (3.20 ± 0.55 fold over GST, n=3) compared to the 14-3-3ζ isoform (2.28 ± 0.51 fold over GST, n=3) but this was not significant. Whilst PLD1 has the motif that more closely matches the optimal consensus target for 14-3-3 association, the binding *in vitro* does not seem to indicate a higher affinity for PLD1 over PLD2 compared to GST control levels. The potential 14-3-3 isoform specificity arising from the PLD2 binding may arise from the less than optimal PLD2 binding motif. It has been suggested that the 14-3-3 isoforms bind specifically to optimum target consensus sequences with little difference in affinity (as the amphipathic groove is highly conserved amongst all 14-3-3 isoforms), although isoform specificity may become apparent as the potential 14-3-3 recognition motif deviates from that of the optimum RSxpSxP consensus (Yaffe *et al.*, 1997; Aitken *et al.*, 2002). PLD1 contains an optimal high stringency mode-1 14-3-3

Figure 5.2



PLD1 and PLD2 associate with 14-3-3 isoforms *in vitro*.

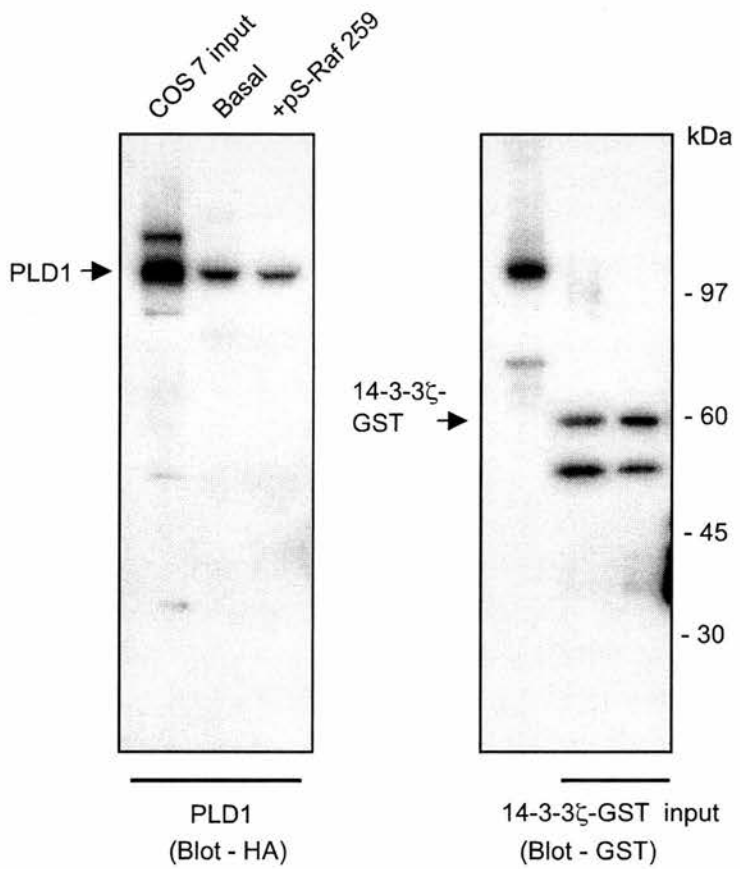
COS 7 cell extracts, transiently overexpressing HA-tagged PLD1 and PLD2 isoforms were incubated with GST-fusion protein constructs of full length isoforms of 14-3-3ζ and 14-3-3η on a glutathione sepharose matrix. The immunoblots show the specific binding of both PLD1 and PLD2 to the GST-14-3-3 constructs above control levels (GST alone).

interacting domain (if indeed this is where 14-3-3 binds) whereas that in PLD2 shows some differences, so 14-3-3/PLD2 interaction may be more dependent upon isoform variation than 14-3-3/PLD1 interaction. This may explain why 14-3-3ζ interacts less well with PLD2 than 14-3-3η does *in vitro*.

The interaction of PLD with 14-3-3 isoforms may be via the 14-3-3 amphipathic groove

The GST-fusion binding assay was repeated using COS 7 cell lysate enriched with HA-PLD1 and this was incubated with the GST-fusion construct of 14-3-3ζ in the presence or absence of 100 μM of the pS-Raf 259 phosphoserine motif blocking peptide. PLD1 was chosen as it has the more distinct 14-3-3 recognition motif. The bound proteins were lysed, separated by SDS-PAGE, Western blotted and visualised using anti-HA HRP-conjugated primary antibody (Roche Diagnostics) (Figure 5.3). The presence of the pS-Raf 259 peptide in the incubation attenuated the binding of PLD1 to the 14-3-3ζ construct. Analysis revealed that the band grey scale density of the pS-Raf 259 peptide incubated condition was 67 ± 8% of the density of basal association of PLD1 (n=2). In comparison, the density of HA-PLD1 associated with the GST control condition was 53 ± 20% of that for the GST-14-3-3ζ construct (from previous data, n=4). Whilst the concentration of 100 μM pS-Raf 259 should, in principle, be sufficient to saturate the GST-14-3-3 in an *in vitro* binding assay (which may be up to 10 mg/ml total GST-fusion protein bound), the incubation conditions using the pS-Raf 259 peptide may not be optimal to efficiently bind to all of the immobilised GST-14-3-3. Thus, a certain amount of GST-14-3-3 could still be available for interaction and may bind cellular partners, including PLD. Nevertheless, the reduction in the pS-Raf 259 treated incubation suggests that competitive inhibition of the 14-3-3 binding site attenuates 14-3-3ζ and PLD1 association, implying that binding is specific and occurs in the amphipathic groove of the 14-3-3 dimer.

Figure 5.3



The pS-Raf 259 peptide partially attenuates PLD and 14-3-3 association.

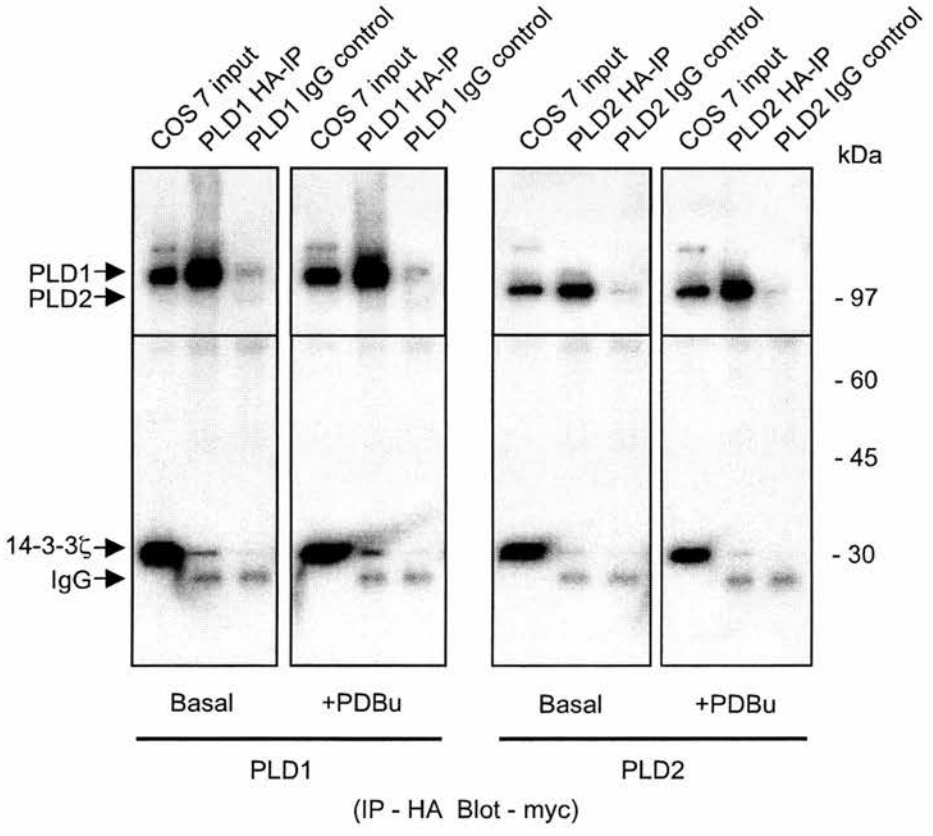
COS 7 cell extracts, transiently overexpressing HA tagged PLD1 were applied to GST-fusion constructs of 14-3-3ζ in the presence or absence of the phosphoserine motif blocking peptide pS-Raf 259. The presence of the pS-259 peptide partially attenuates PLD association with 14-3-3, indicating that it is a competitive blocker of the interaction site.

14-3-3 isoforms co-immunoprecipitate with PLD1 and PLD2

Co-immunoprecipitation studies were carried out to determine whether there might be isoform specificity in the association of PLD isozymes with 14-3-3 isoforms and to determine whether PKC stimulation of PLD had any effect on the potential association. HA-PLD1 or HA-PLD2 and the myc-tagged 14-3-3ζ isoform (14-3-3ζ-myc) cDNAs were transiently transfected into COS 7 cells in a 1:1 ratio. The cells were serum-deprived for 16 hours and stimulated where necessary with 500 nM phorbol 12,13-dibutyrate for 10 mins and then lysed. The PLD was pulled down using immunoprecipitation directed against the HA tag (1 μg/ml mouse monoclonal 12CA5 or 1 μg/ml rat monoclonal 9E10, Roche Diagnostics). Non-immune mouse or rat IgG (1 μg/ml) was used as a negative control. The immunoprecipitates of the HA-PLD were lysed from the beads, separated by SDS-PAGE, Western blotted and the co-immunoprecipitated levels of 14-3-3ζ were detected using rabbit polyclonal anti c-myc antibody (Upstate Biotech, Milton Keynes, UK) followed by anti-rabbit HRP-conjugated secondary antibody (Chemicon, Hants, UK) and standard ECL detection (Figure 5.4). The input levels of immunoprecipitated PLD were visualised with rat anti-HA HRP-conjugated primary antibody (Roche Diagnostics) and are also shown, at top.

The 14-3-3ζ isoform co-immunoprecipitated specifically with both PLD1 and PLD2 above IgG control levels under basal conditions, supporting the findings of the *in vitro* GST-binding assay. Upon PKC-mediated PLD activation by phorbol dibutyrate, the levels of 14-3-3ζ that were co-immunoprecipitated with PLD1 were not significantly changed from the levels of basal association (1.33 ± 0.39 fold over basal, n=3). The levels of 14-3-3ζ that were co-immunoprecipitated with PLD2 upon PKC stimulation were also not significantly altered from basal levels (0.84 ± 0.27 fold of basal, n=3).

Figure 5.4



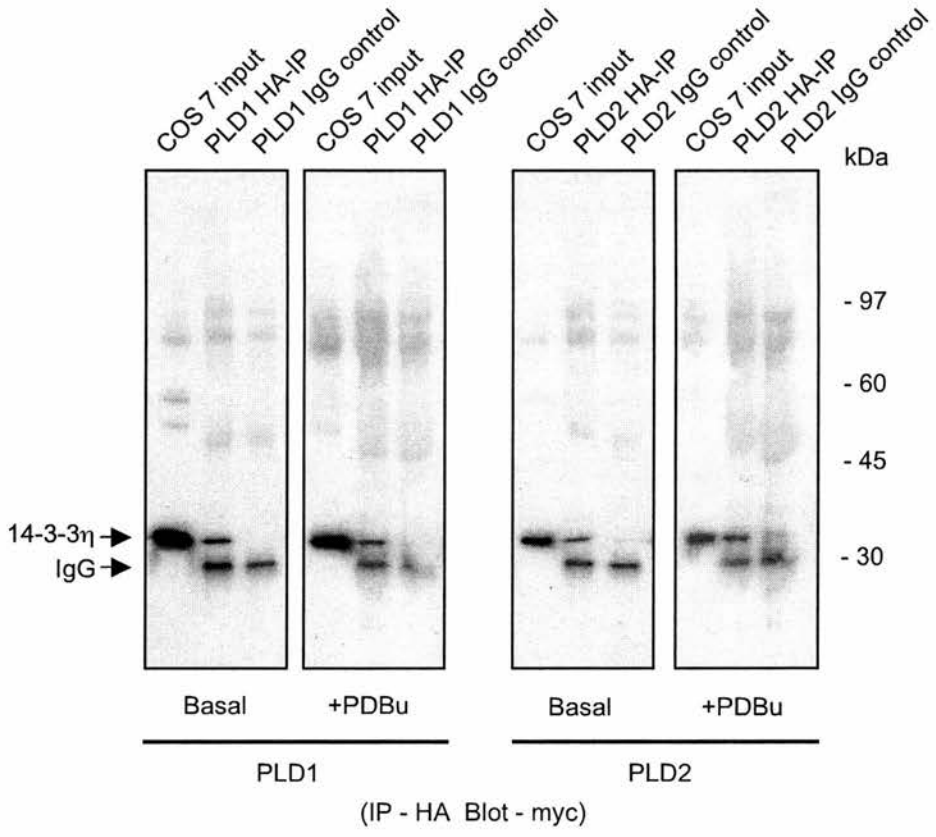
PLD1 and PLD2 co-immunoprecipitate with the 14-3-3ζ isoform.

COS 7 cells, transiently overexpressing 14-3-3ζ-myc and HA-PLD1 or HA-PLD2 were left in a basal state or stimulated with 500 nM PDBu for 10 minutes. Immunoprecipitates were directed against the HA tag (IP) and co-immunoprecipitated 14-3-3 visualised by blotting for the myc tag. Non-immune IgG was used as a negative control (IgG). The stimulation of PKC by PDBu does not appreciably affect 14-3-3ζ association with PLD1 or with PLD2. The immunoprecipitation of the HA levels is also shown (PLD1 and PLD2).

Similar experiments were then carried out, transiently co-transfecting COS 7 cells with HA-PLD1 or HA-PLD2 and myc-14-3-3η. The cells were serum deprived for 16 hours and stimulated where necessary with 500 nM PDBu for 10 mins. The HA tag of the PLDs were immunoprecipitated from the cellular lysates using monoclonal antibodies 12CA5 or 9E10 (Roche Diagnostics) as before and co-immunoprecipitated proteins were lysed, separated by SDS-PAGE and Western blotted. The levels of 14-3-3η that co-immunoprecipitated with PLD1 and PLD2 were visualised using rabbit polyclonal anti-myc primary antibody (Upstate), followed by HRP-conjugated anti-rabbit secondary antibody (Chemicon) and standard ECL detection (Figure 5.5). Under basal conditions, the 14-3-3η isoform associated with both PLD1 and PLD2 above IgG controls. The levels of 14-3-3η that co-immunoprecipitated with PLD1 were not affected by PKC stimulation (0.98 ± 0.16 fold of basal, n=3). The levels of 14-3-3η that were co-immunoprecipitated with PLD2 were also not discernibly affected by PKC stimulation (1.29 ± 0.21 fold over basal, n=3).

Finally, further co-immunoprecipitation studies were carried out with the 14-3-3ε isoform and HA-PLD1 or HA-PLD2 in COS 7 cells. The cells were transfected, stimulated with PDBu and the lysates immunoprecipitated for the HA tag as before (clone 9E10 or 12CA5, Roche). The co-immunoprecipitated proteins were separated by SDS-PAGE, Western blotted and the 14-3-3ε levels were visualised as previously (Figure 5.6). The basal co-immunoprecipitation level of 14-3-3ε with PLD1 was consistently only slightly above the IgG control level, as was the co-immunoprecipitation level of 14-3-3ε with PLD2 (indicating a relatively low level of association of 14-3-3ε with both isozymes under basal

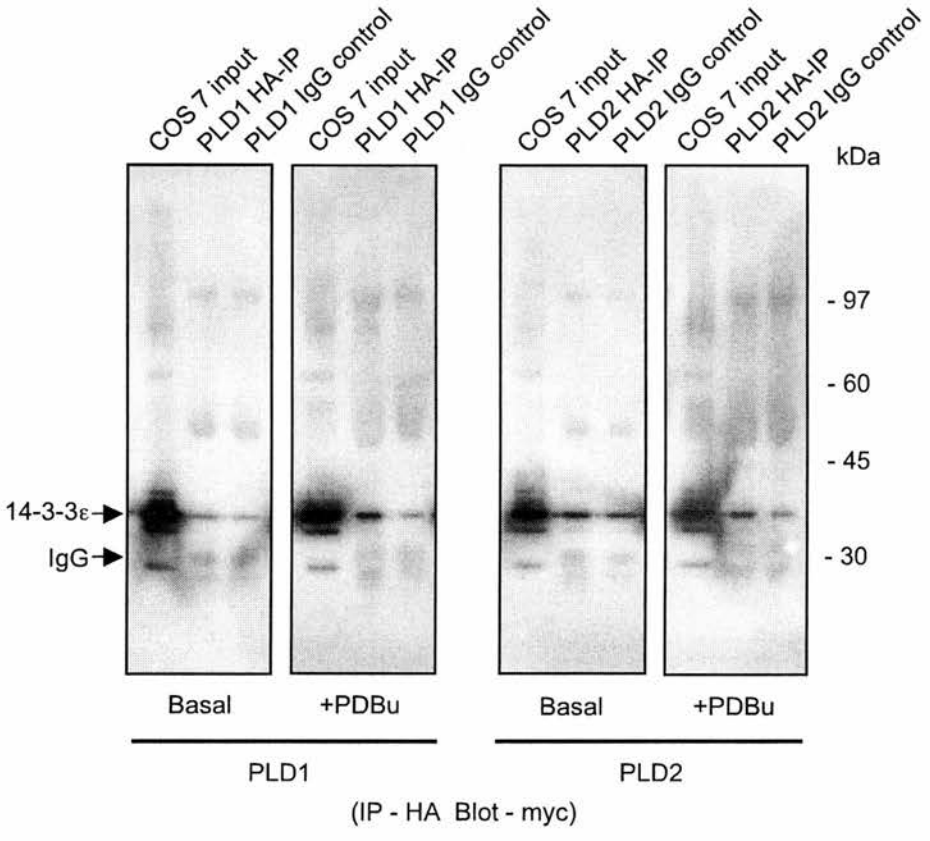
Figure 5.5



PLD1 and PLD2 co-immunoprecipitate with the 14-3-3η isoform.

COS 7 cells, transiently overexpressing myc-14-3-3ζ and HA-PLD1 or HA-PLD2 were left in a basal state or stimulated with 500 nM PDBu for 10 minutes. Immunoprecipitates were directed against the HA tag (IP) and co-immunoprecipitated 14-3-3 visualised by blotting for the myc tag. Non immune IgG was used as a negative control (IgG). The stimulation of PKC by PDBu does not markedly affect 14-3-3η association with PLD1 or with PLD2.

Figure 5.6



PLD1 and PLD2 co-immunoprecipitate with the 14-3-3ε isoform.

COS 7 cells, transiently overexpressing 14-3-3ε-myc and HA-PLD1 or HA-PLD2 were left in a basal state or stimulated with 500 nM PDBu for 10 minutes. Immunoprecipitates were directed against the HA tag (IP) and co-immunoprecipitated 14-3-3 visualised by blotting for the myc tag. Non immune IgG was used as a negative control (IgG). The stimulation of PKC by PDBu may enhance 14-3-3ε association with PLD1, but does not affect 14-3-3ε association with PLD2.

conditions). Upon PKC stimulation, the level of associated 14-3-3ε with PLD1 appeared to increase over basal (1.84 ± 0.55 fold over basal, n=3), compared to PLD2 where no difference was observed (1.08 ± 0.10 fold over basal, n=3) but the increase in association of PLD1 and 14-3-3ε observed upon PKC stimulation was not statistically significant.

A point to note is that the 14-3-3 dimers formed when overexpressing the isoforms may be either homo- or heterodimers (for example 14-3-3ε has a tendency to only form heterodimers *in vivo*) (Jones *et al.*, 1995a; Chaudhri *et al.*, 2003). The nature of the 14-3-3 dimer itself may have implications for the isoform specificity and function, as although the detection of the transfected isoforms are indicative of their presence in the interaction, it is difficult to determine which other isoform may be present in the dimer formed. Furthermore, the results make no representation about the endogenous levels of 14-3-3 that may be docked to native PLD and whether the *de novo* interaction between the tagged proteins accurately reflects the situation in the physiological system or has been able to displace preformed native complexes. Nevertheless, a potential isoform specificity indicated by the result suggests a possible level of regulation of interaction, the reasons for which may be functionally important.

The stimulation of PKC alters colocalisation of PLD and 14-3-3ζ

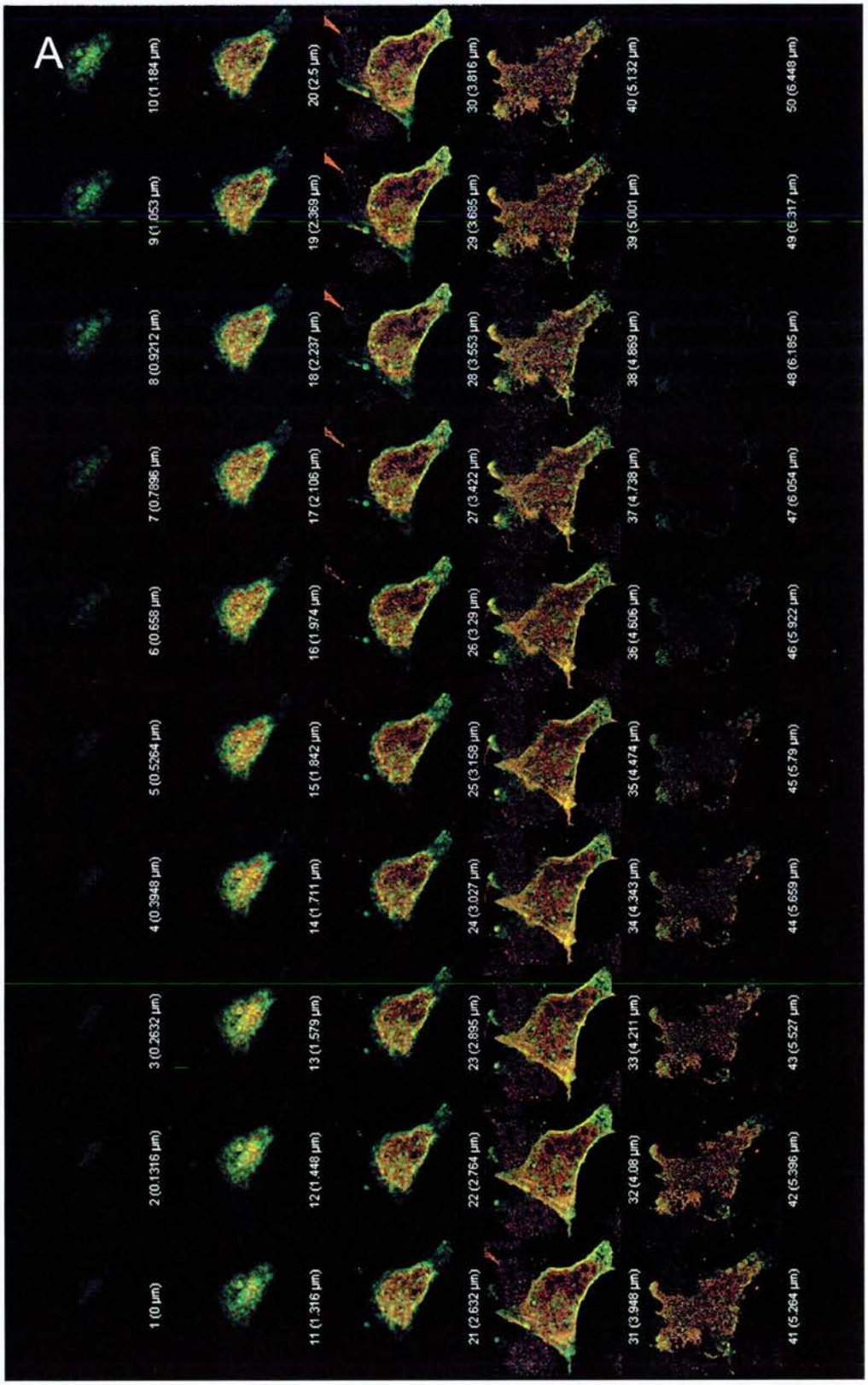
To ascertain whether the subcellular localisation of PLD1 and the isoforms of 14-3-3 were similar, HA-PLD1 and myc tagged 14-3-3ζ cDNAs were transfected into COS 7 cells at a 1:1 ratio. The cells were grown on coverslips and were stimulated where necessary by activating protein kinase C with 500 nM phorbol 12,13-dibutyrate (PDBu) for 10 minutes. They were permeabilised and fixed, and stained with mouse anti-HA primary antibody clone 12CA5 (Roche diagnostics, East Sussex, UK) and rabbit anti-myc primary antibody (Upstate, UK) followed by Alexa Fluor goat anti-mouse 488 nm and anti-rabbit 567 nm secondary antibodies (Molecular Probes, Leiden, The Netherlands). The visualisations of COS 7 cells containing PLD1 (green) and 14-3-3ζ (red) are shown in Figure 5.7.

In unstimulated cells, PLD1 localised to punctate structures, perinuclear compartments and the plasma membrane as found previously. The 14-3-3ζ isoform appeared to localise throughout the cells, and was present in higher levels in the cytosolic compartment, but was also expressed in the nucleus. A small proportion of 14-3-3ζ was observed at the plasma membrane. This distribution of 14-3-3ζ was found to be similar to that of the live-cell investigations made by van Hemert and colleagues in variety of cell types (van Hemert *et al.*, 2004). Upon PKC stimulation, the majority of PLD1 translocated from the intracellular compartments to the plasma membrane and 14-3-3ζ appeared to migrate from the nuclear compartment to the cytosolic or plasma membrane compartments. Colocalisation was measured as in chapter 4 and the mean coefficient of correlation of both channels in unstimulated cells was determined to be 0.12 ± 0.04 (n=4) and in PDBu stimulated cells this value increased to 0.26 ± 0.06 (n=6) but this was not significant ($p>0.05$ Unpaired Student t-test).

The experiment was repeated for PLD2 (green) and 14-3-3ζ (red) and the visualisations of both basal and stimulated cells are shown in Figure 5.8. In unstimulated cells, PLD2 was primarily localised to the plasma membrane and 14-3-3ζ had a similar

distribution to that found previously. Upon PKC stimulation, 14-3-3ζ appeared to migrate from the nucleus to the cytosol as before (with a small increase at the plasma membrane). The localisation of PLD2 did not markedly change upon PKC activation, except that a small proportion migrated into the cell. The values for the colocalisation were measured and the mean colocalisation coefficient of correlation in unstimulated cells was determined to be 0.06 ± 0.04 (n=4) and in stimulated cells was 0.15 ± 0.10 (n=3) with no significant difference ($p>0.05$, Unpaired Student t-test). These results suggest that PKC stimulation does not result in any significant increase in colocalisation of either of the PLD isozymes with 14-3-3ζ.

Figure 5.7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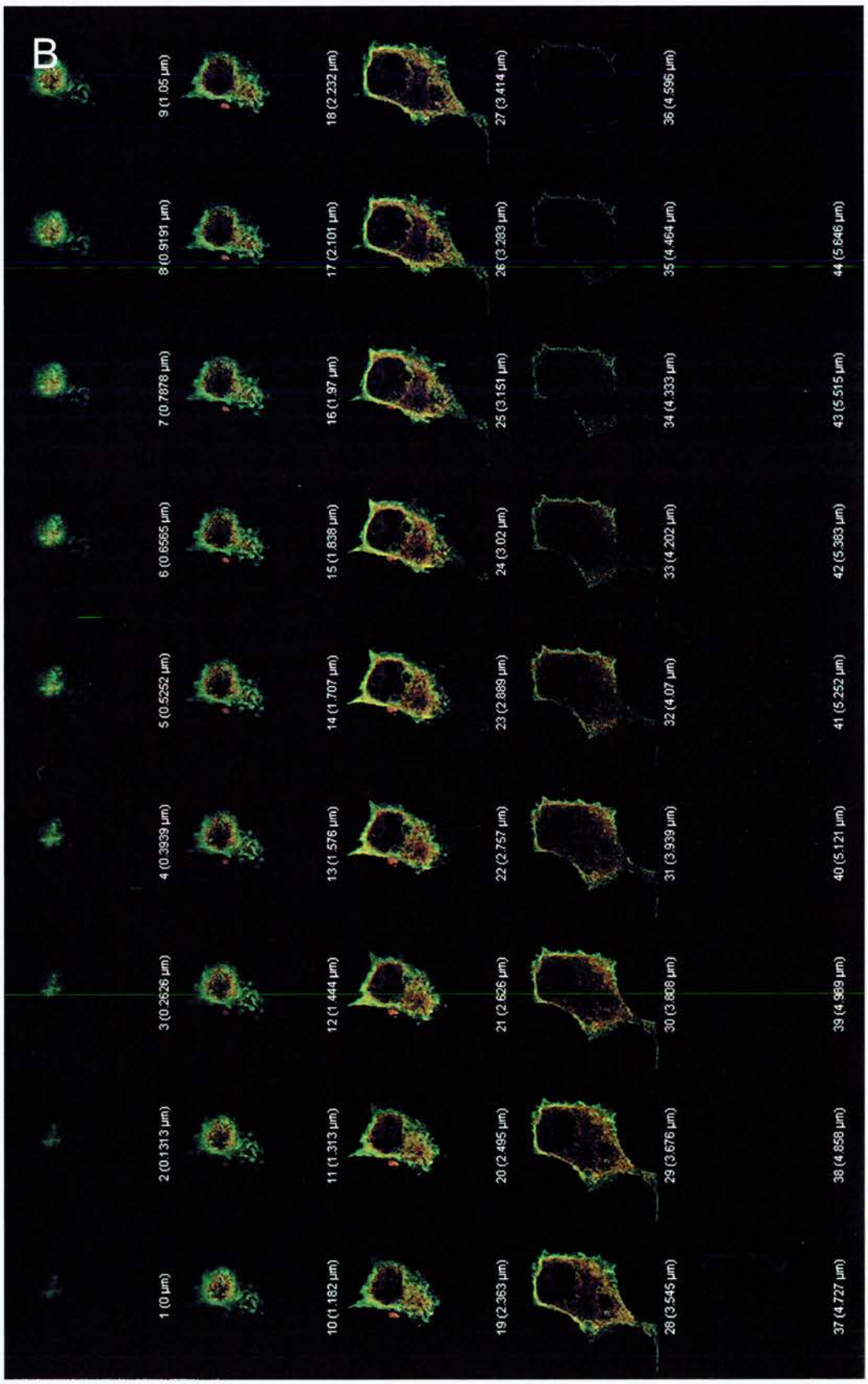
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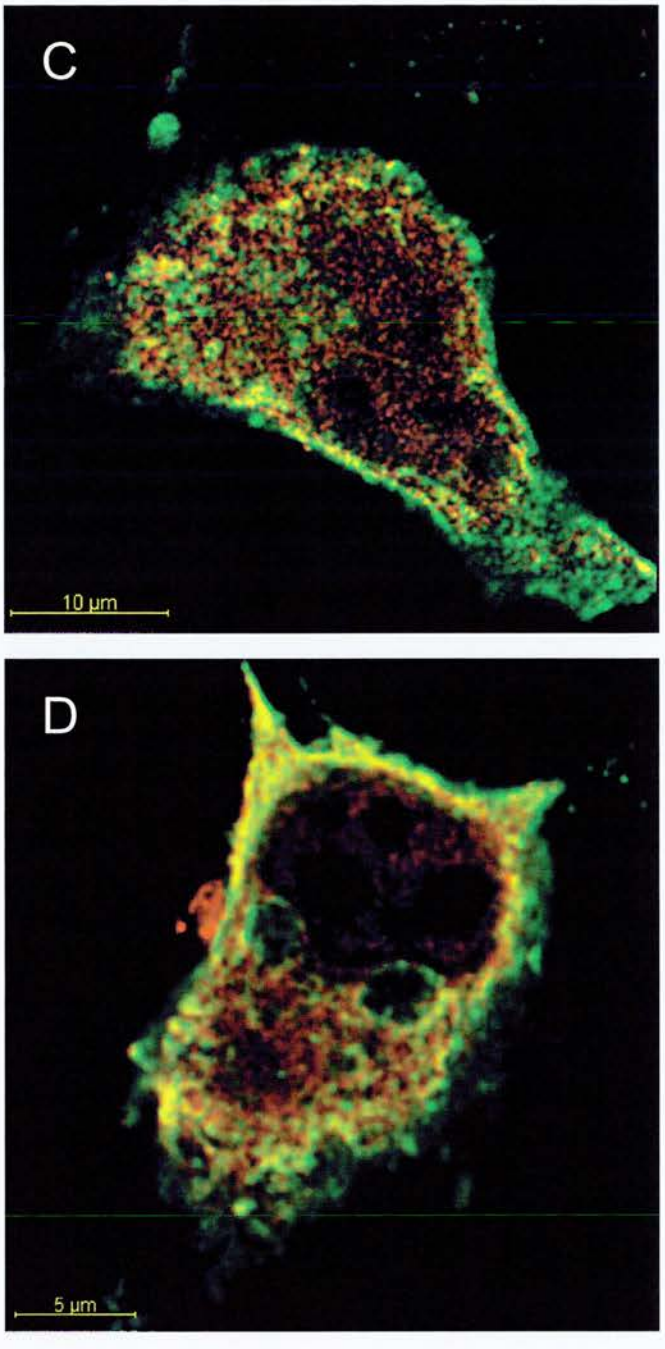
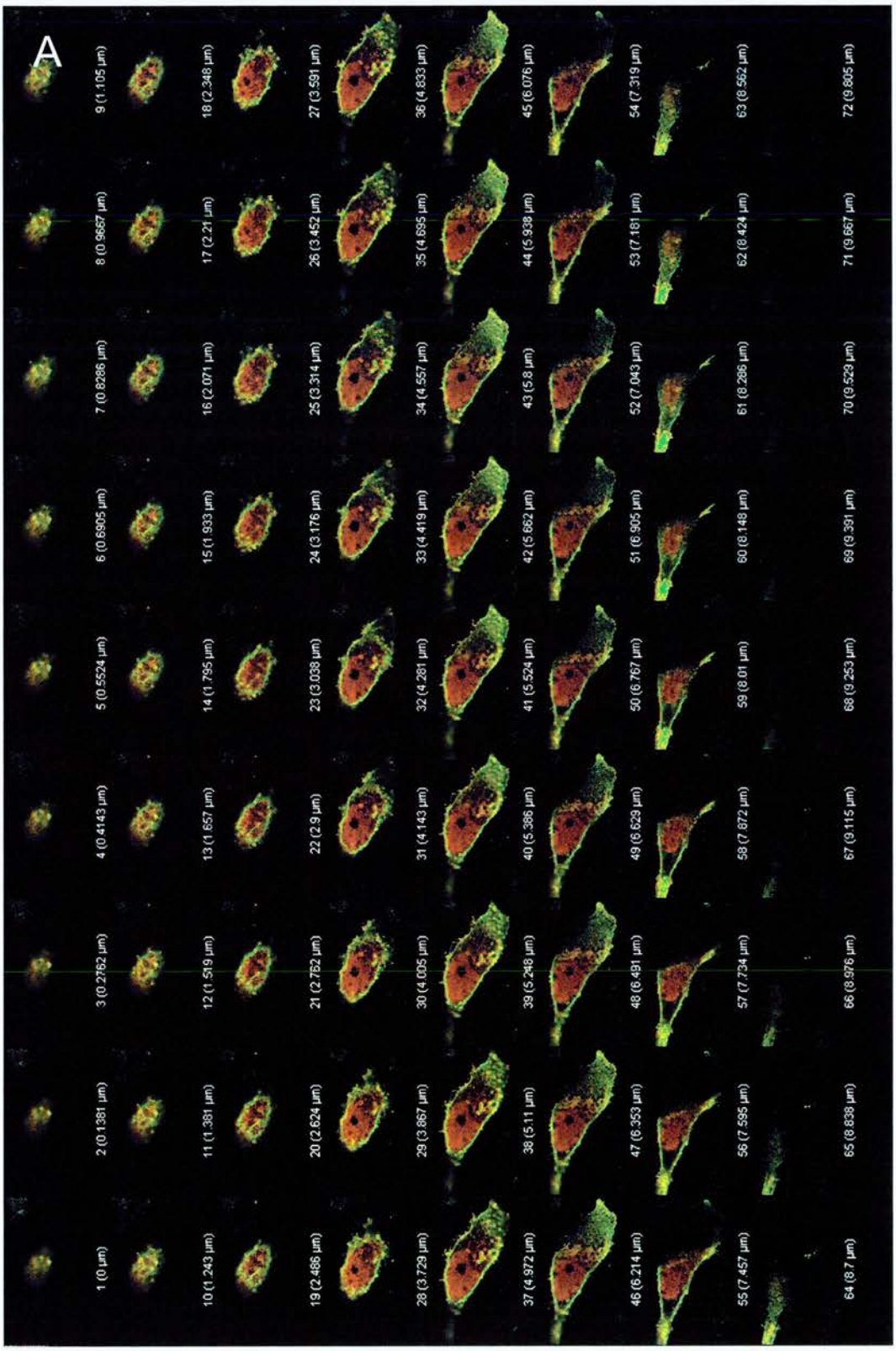


The effect of PKC stimulation on the localisation of PLD1 and 14-3-3ζ.

The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD1 (green) and 14-3-3ζ-myc (red).

Figure 5.8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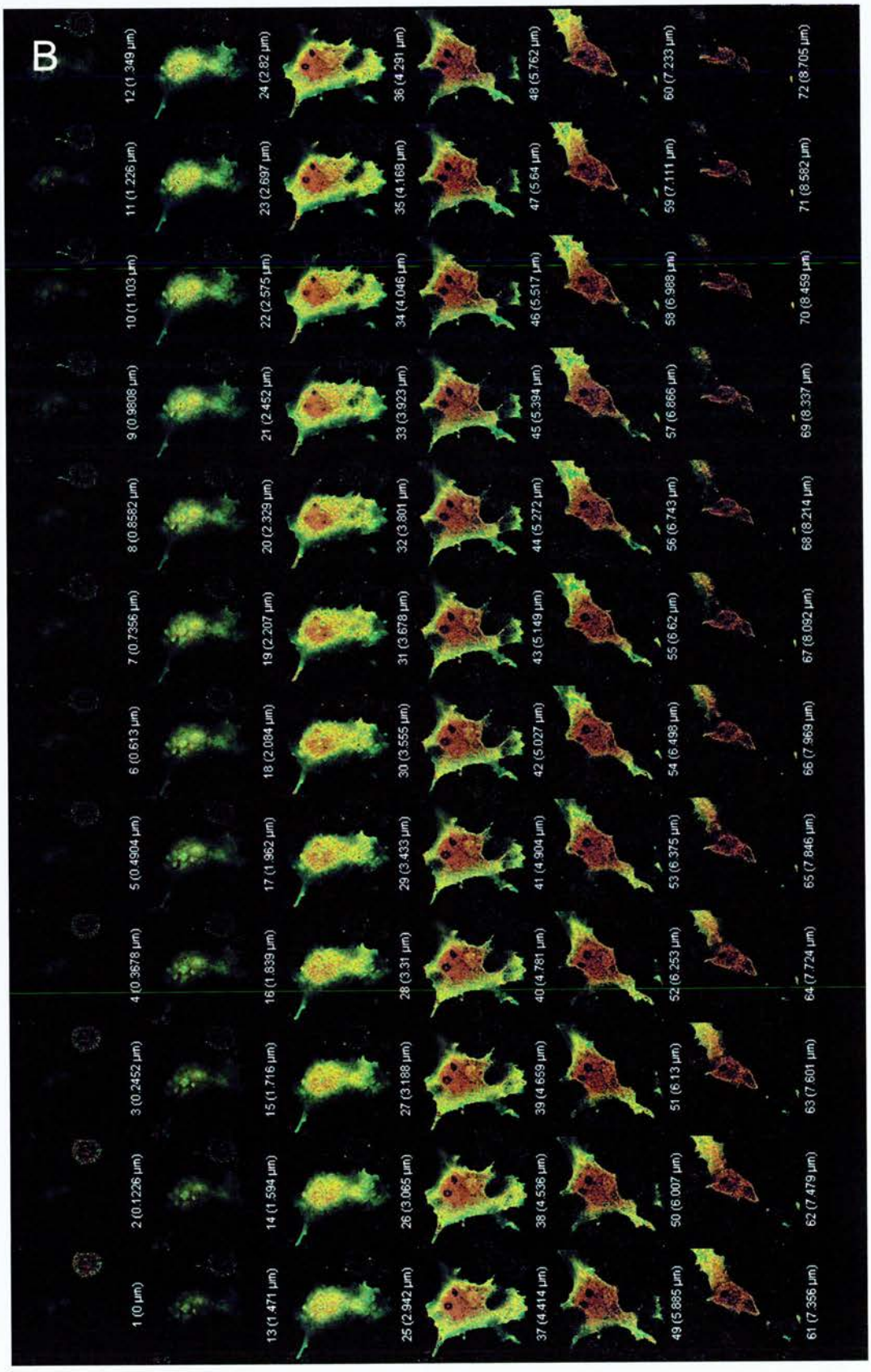
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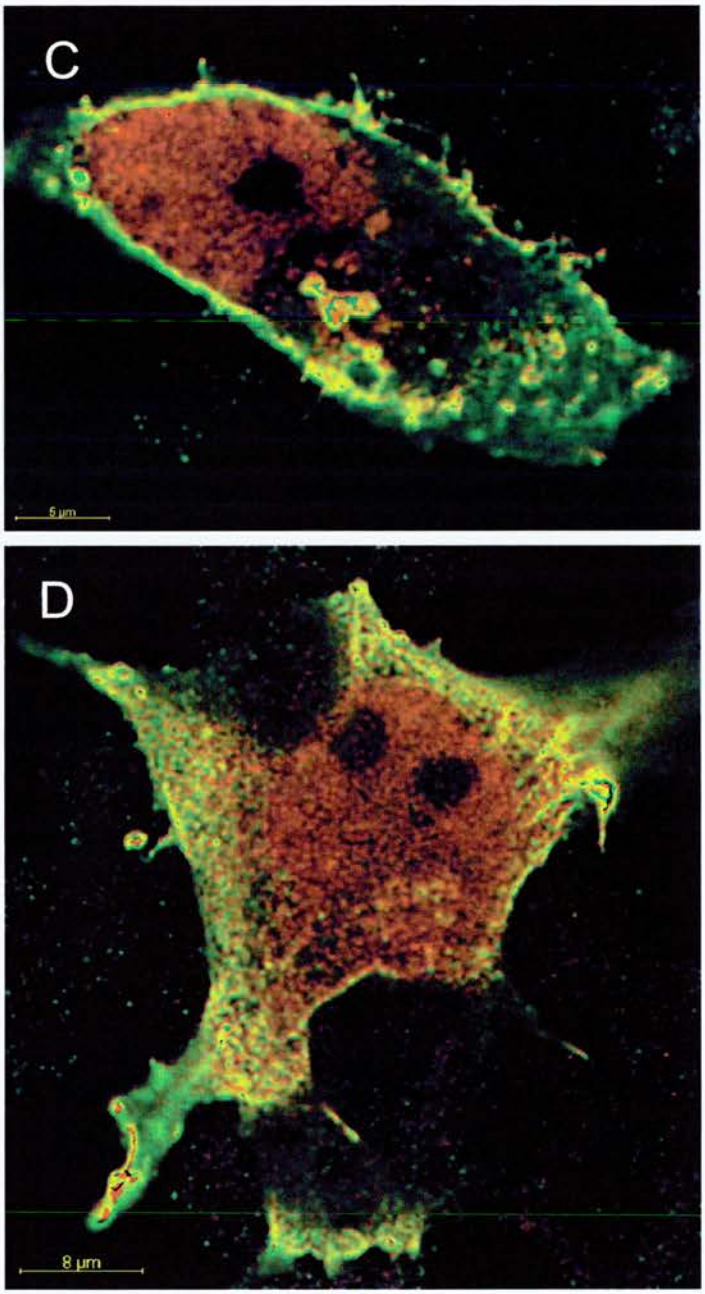


The effect of PKC stimulation on the localisation of PLD2 and 14-3-3ζ.

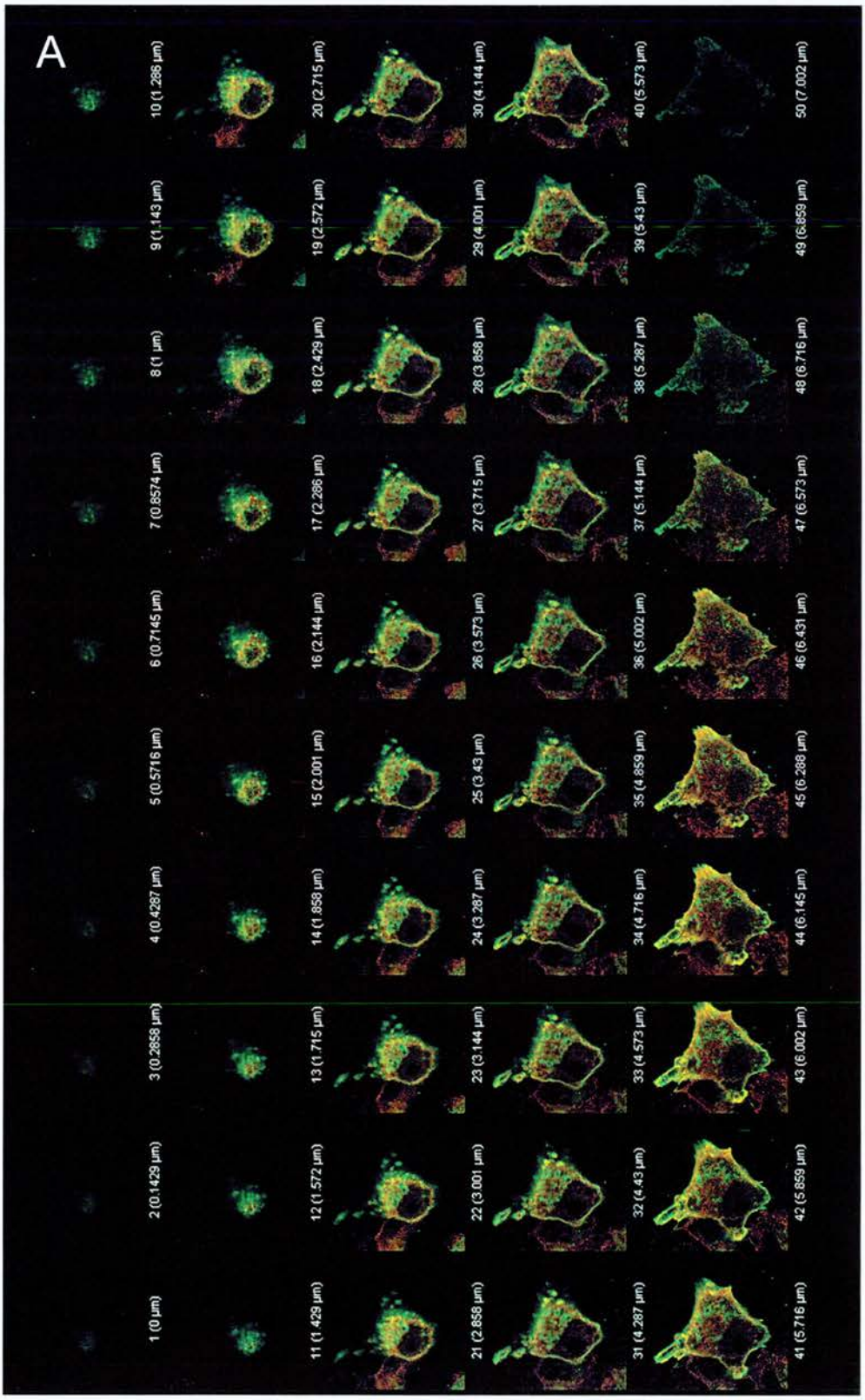
The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD2 (green) and 14-3-3ζ-myc (red).

The stimulation of PKC alters colocalisation of PLD and 14-3-3η

COS 7 cells were transiently transfected with HA-PLD1 and myc-14-3-3η and were stimulated where indicated with 500 nM PDBu for 10 min. The cells were fixed and stained exactly as before. The visualisations of PLD1 (green) and 14-3-3η (red) in unstimulated and PDBu stimulated cells are shown in Figure 5.9. The PLD1 localised to many internal structures as previously. The 14-3-3η isoform appeared to localise primarily to the cytosol and not to the nuclear compartment or the plasma membrane, (this was a similar distribution to that of transfected 14-3-3σ in the study by van Hemert and colleagues (van Hemert *et al.*, 2004)). The mean value for the colocalisation coefficient of correlation in resting cells was determined to be 0.23 ± 0.10 (n=3). Upon PKC stimulation by PDBu, PLD1 migrated to the plasma membrane and the 14-3-3η localisation became more ubiquitous within the cell (migrating into the nucleus). The mean coefficient of correlation value was 0.22 ± 0.6 (n=3), indicating that there was no discernible difference in the levels of colocalisation of PLD1 and 14-3-3η due to PKC-mediated PLD1 activation.

COS 7 cells were also transiently co-transfected with HA-PLD2 and myc-14-3-3η, stimulated where necessary with PDBu and imaged as previously (Figure 5.10). The PLD2 in resting cells was bound exclusively at the plasma membrane and the 14-3-3η, was again localised to the cytosolic compartment (not the nucleus). Upon PDBu stimulation, a small proportion of PLD2 internalised on small punctate structures and localisation of 14-3-3η did not appreciably change, except there was an increase in 14-3-3η at the plasma membrane (most clearly visible on the upper surface of the cell). The mean coefficient of correlation of the 14-3-3η and PLD2 proteins in resting cells was 0.15 ± 0.05 (n=3) and in stimulated cells was 0.32 ± 0.09 (n=4). This change was not quite significant (p=0.054, Unpaired Student t-test), nevertheless it represented a potential increase in colocalisation of 14-3-3η with PLD2.

Figure 5.9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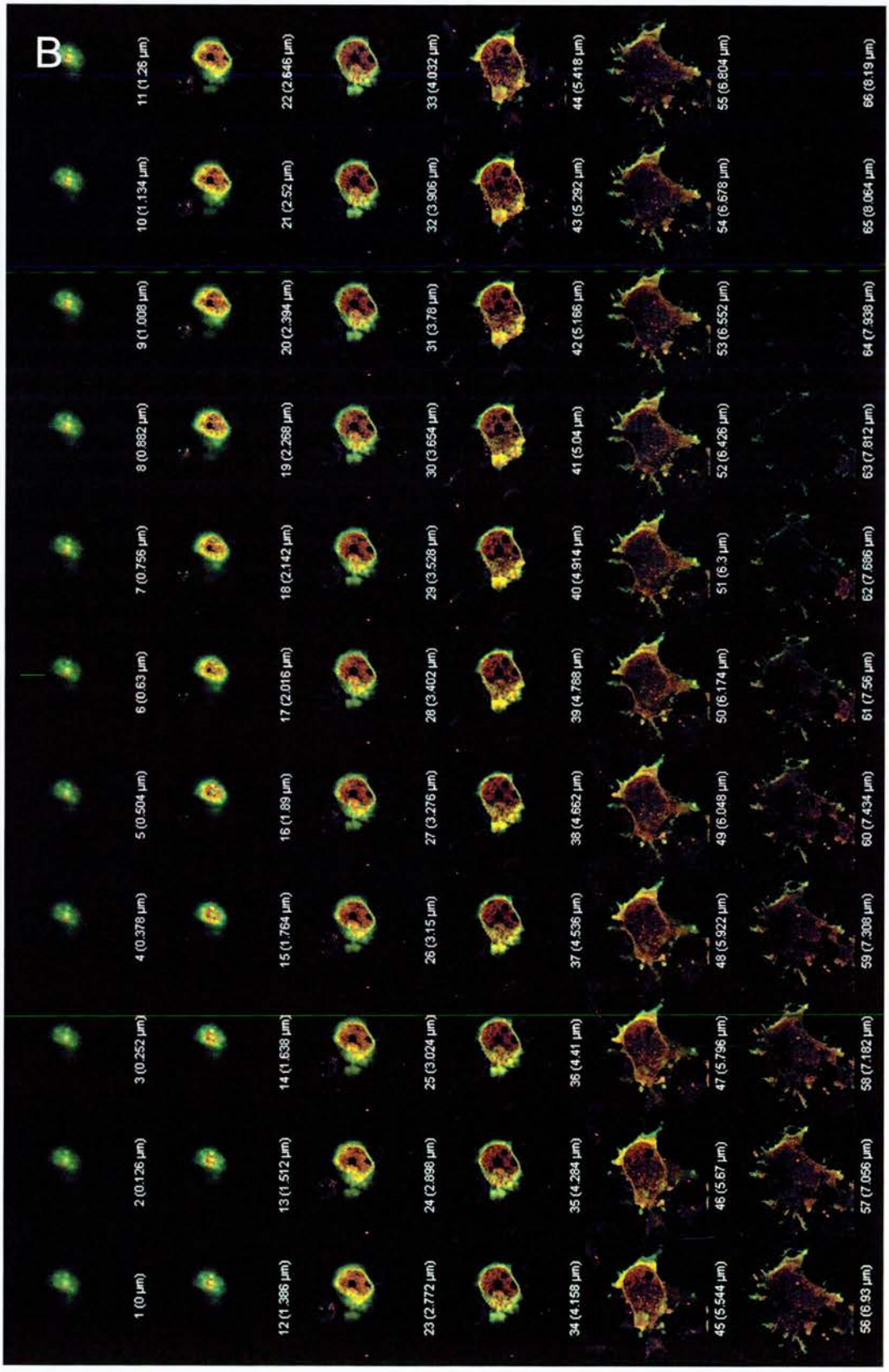
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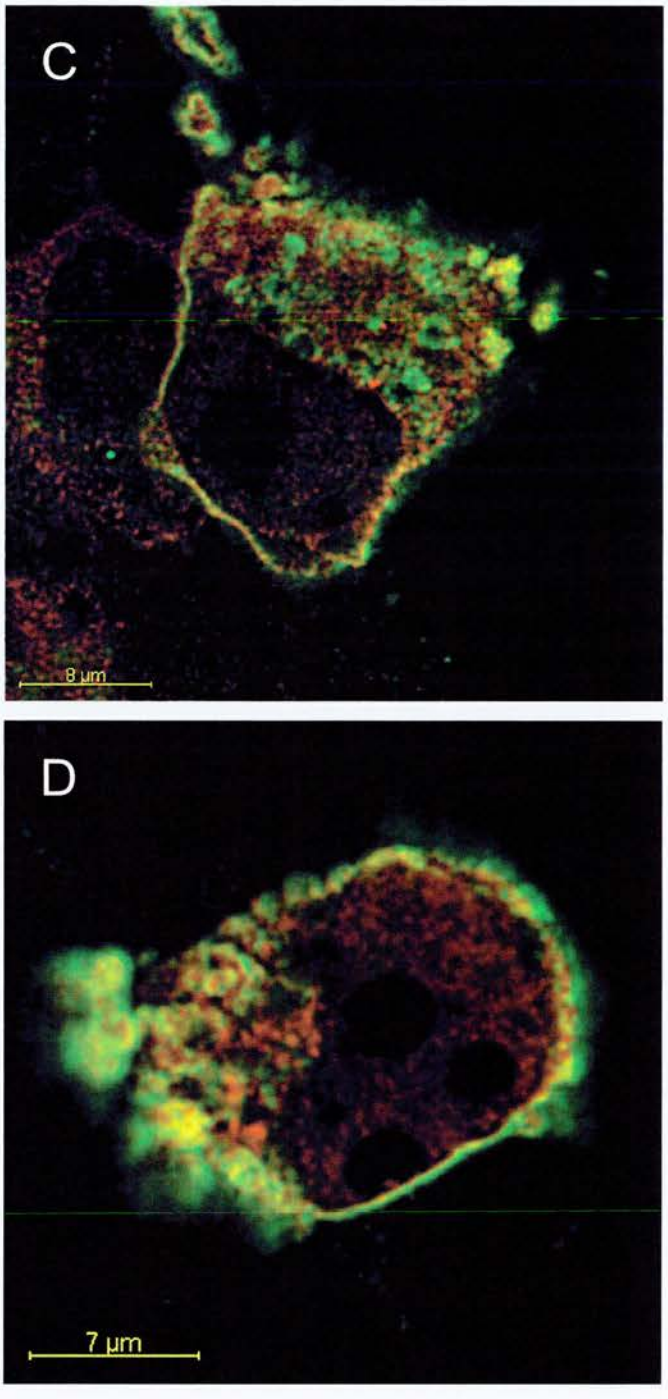
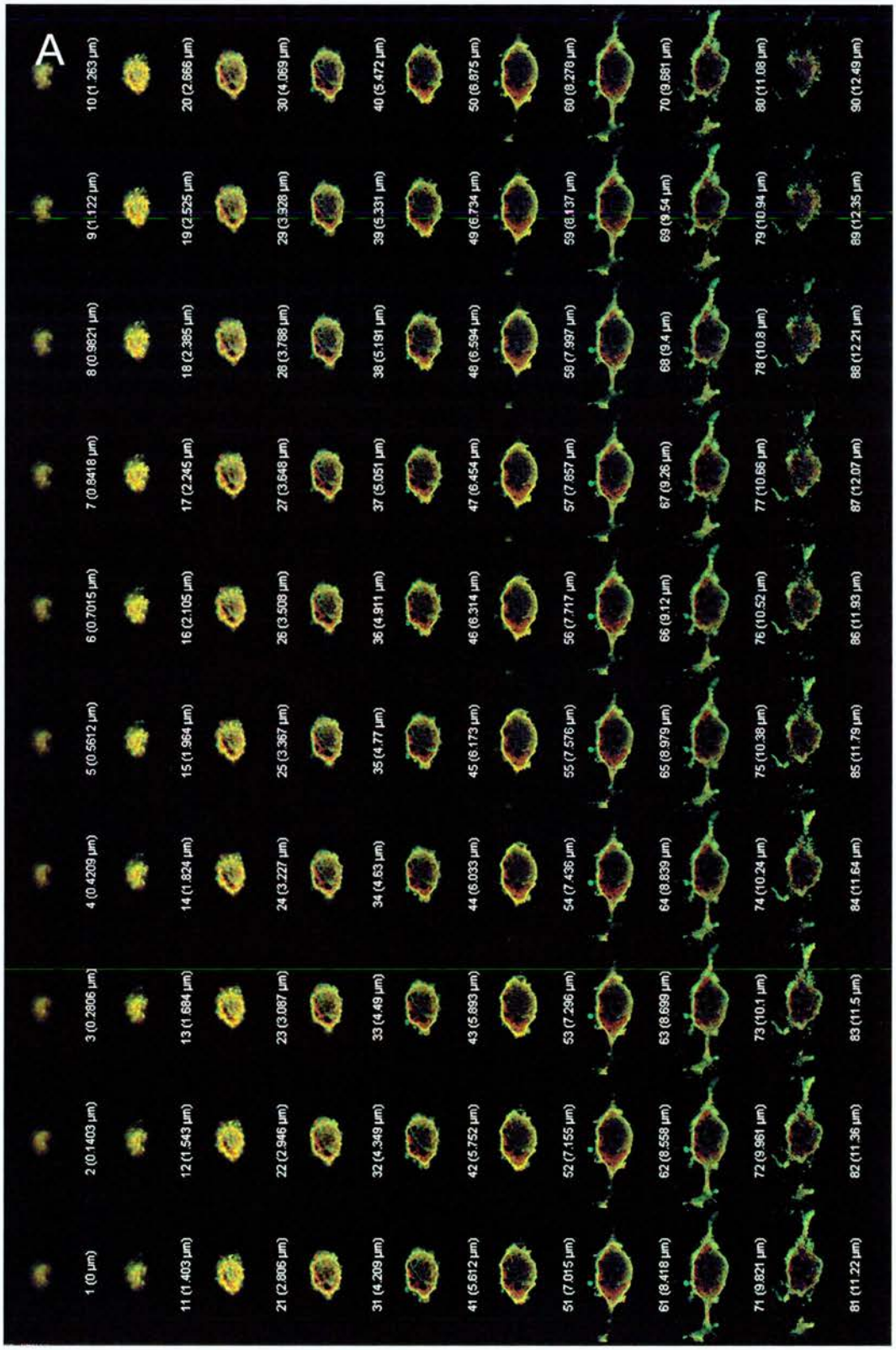


The effect of PKC stimulation on the localisation of PLD1 and 14-3-3η.

The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD1 (green) and myc-14-3-3η (red).

Figure 5.10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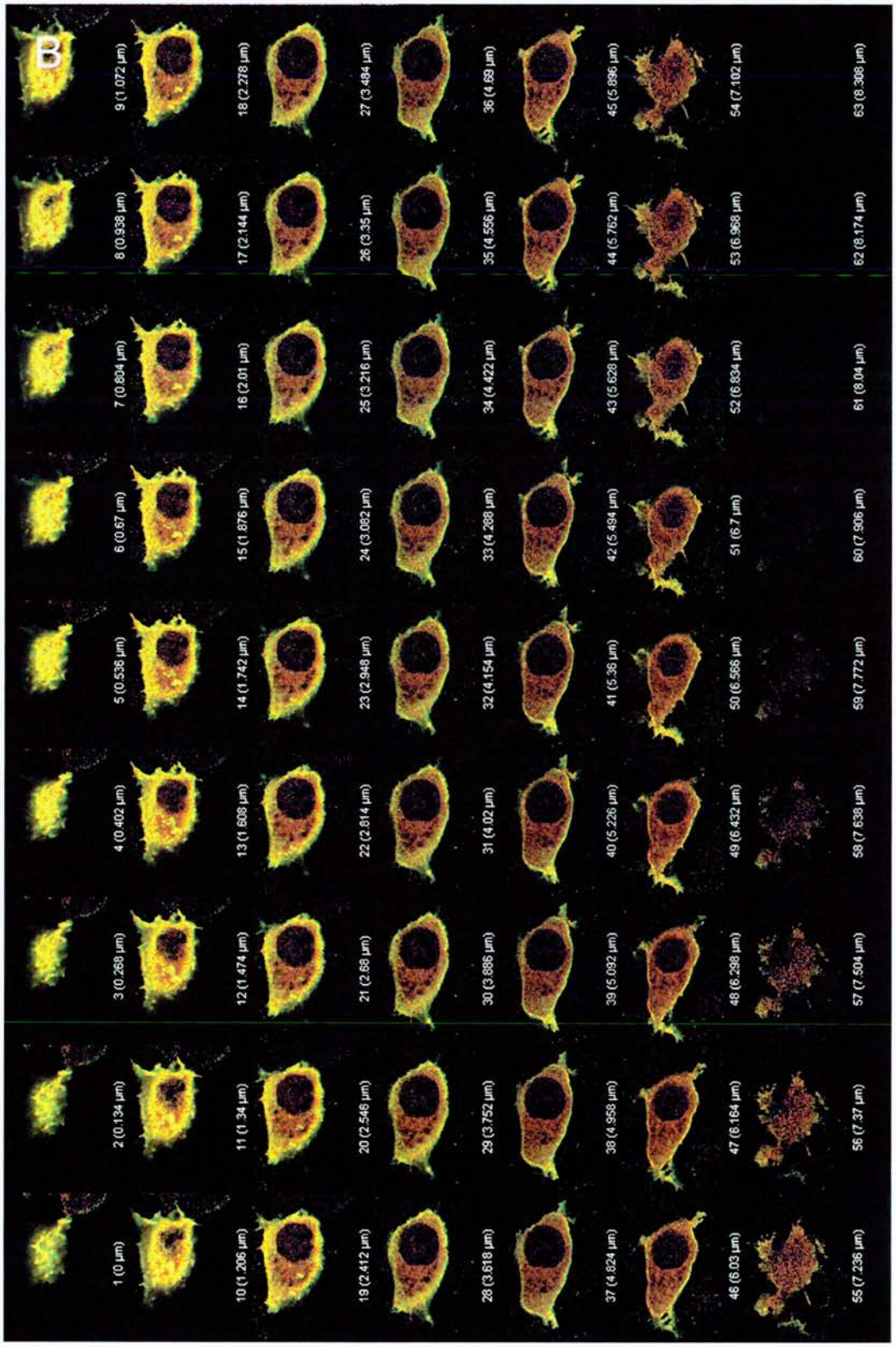
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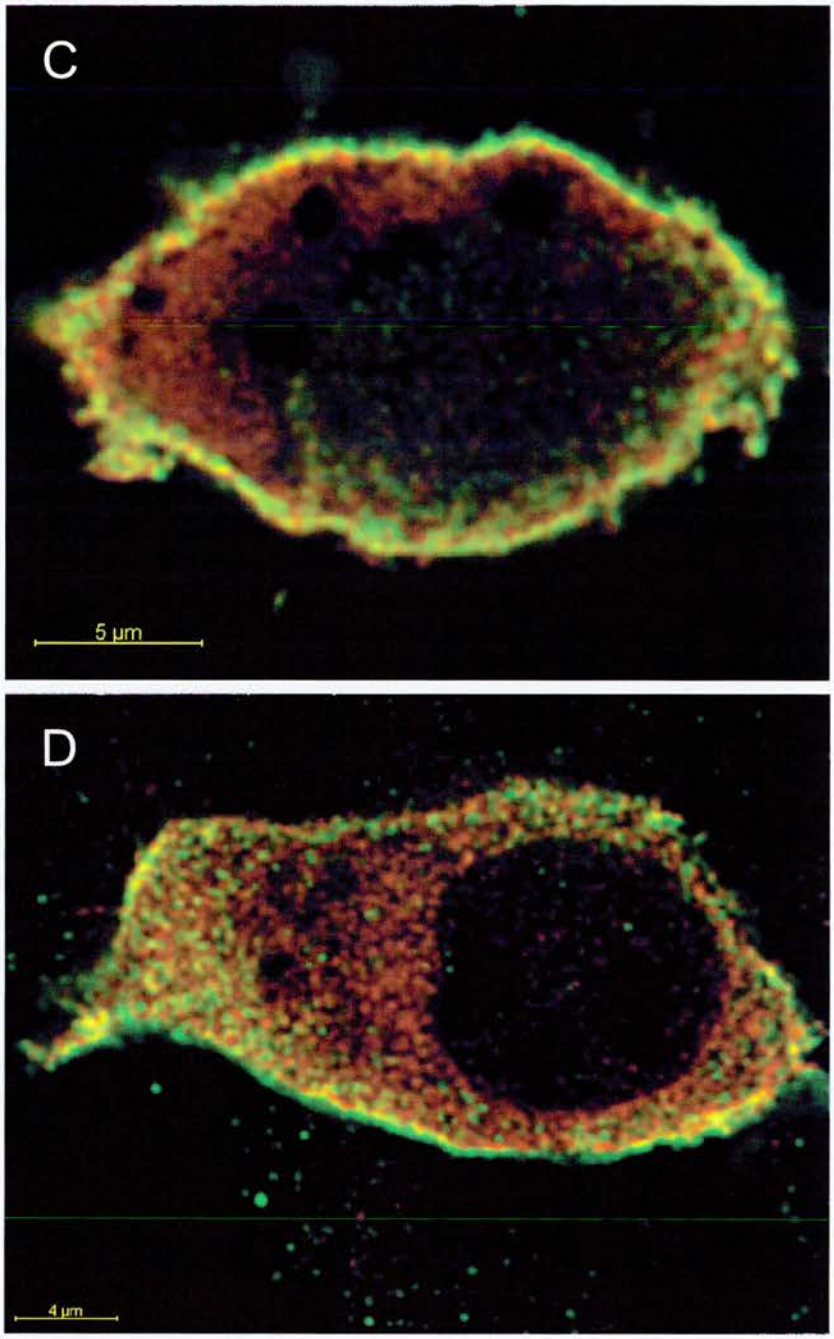


The effect of PKC stimulation on the localisation of PLD2 and 14-3-3η.

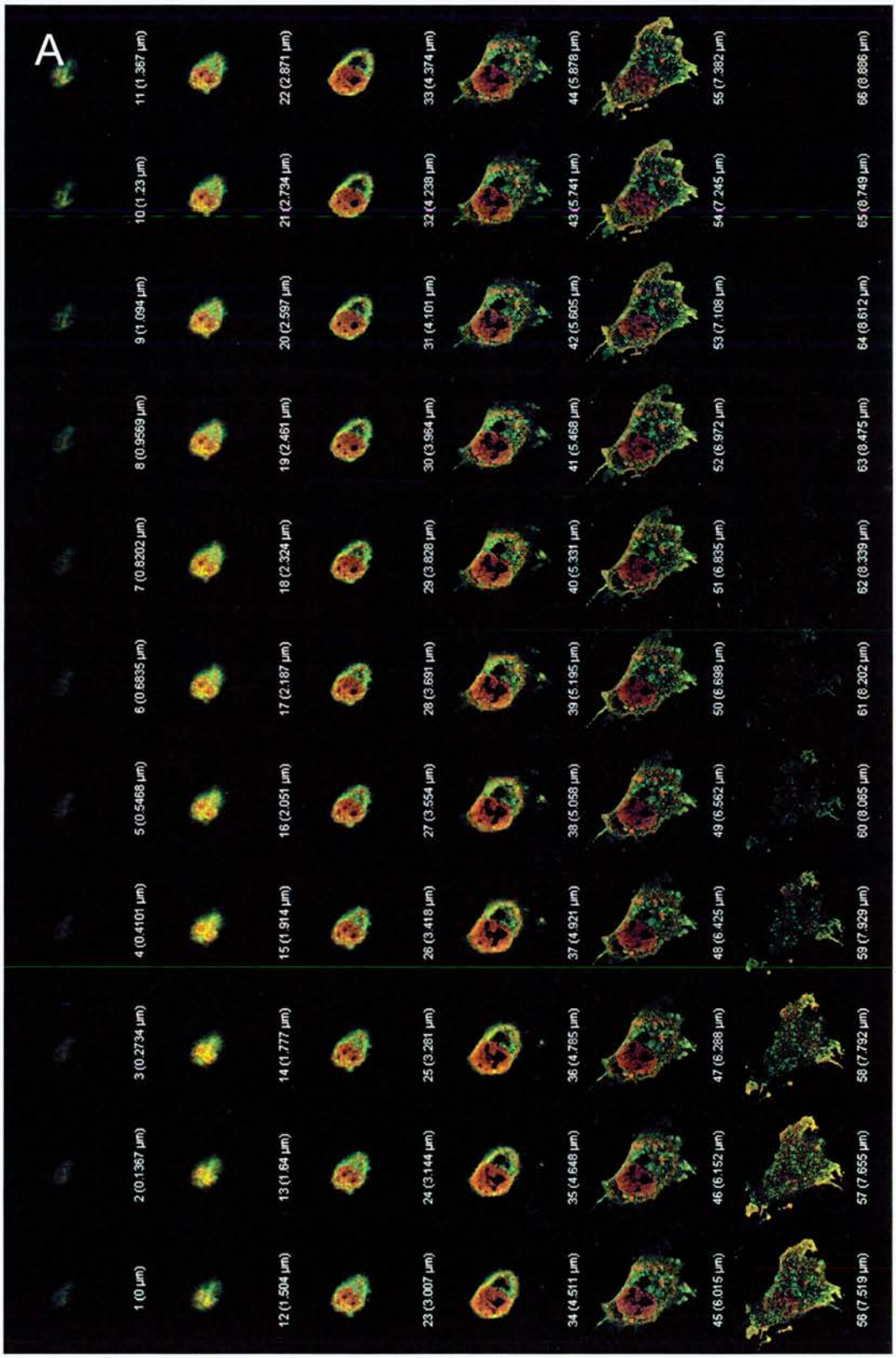
The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD2 (green) and myc-14-3-3η (red).

The stimulation of PKC alters colocalisation of PLD and 14-3-3ε

Imaging data was then collected for COS 7 cells transiently transfected with HA-PLD1 and 14-3-3ε-myc. Unstimulated COS 7 cells were imaged as before and are shown in Figure 5.11. The localisation of PLD1 was similar, predominantly localising to the perinuclear apparatus and forming punctate structures within the cell. The 14-3-3ε isoform displayed a localisation that appeared to be exclusively within the nucleus in most cells. This compartmentalisation meant that there was very little colocalisation under basal conditions, the mean coefficient of correlation was 0.02 ± 0.08 (n=3). Upon PKC stimulation, PLD1 translocated to the plasma membrane as before and 14-3-3ε migrated from the nucleus to the cytosolic compartment of the cell, with a small proportion directed to the plasma membrane. The mean colocalisation coefficient of correlation significantly increased to 0.32 ± 0.02 (n=3) ($p<0.05$, Unpaired student t-test) in PKC stimulated cells.

The experiment was repeated for HA-PLD2 and 14-3-3ε-myc and the visualisation of the cells is shown in Figure 5.12. Under basal conditions, as with PLD1, the distribution of both PLD2 and 14-3-3ε was discrete. PLD2 was localised to the plasma membrane as previously indicated and 14-3-3ε was predominantly localised within the nucleus. The colocalisation coefficient of correlation was 0.13 ± 0.07 (n=3). Upon stimulation PLD2 showed a small internalisation and 14-3-3ε appeared to migrate out of the nuclear compartment, however the colocalisation coefficient was reduced to 0.02 ± 0.06 (n=2). This value is unexpectedly low compared to the basal value and may be validated with additional data.

Figure 5.11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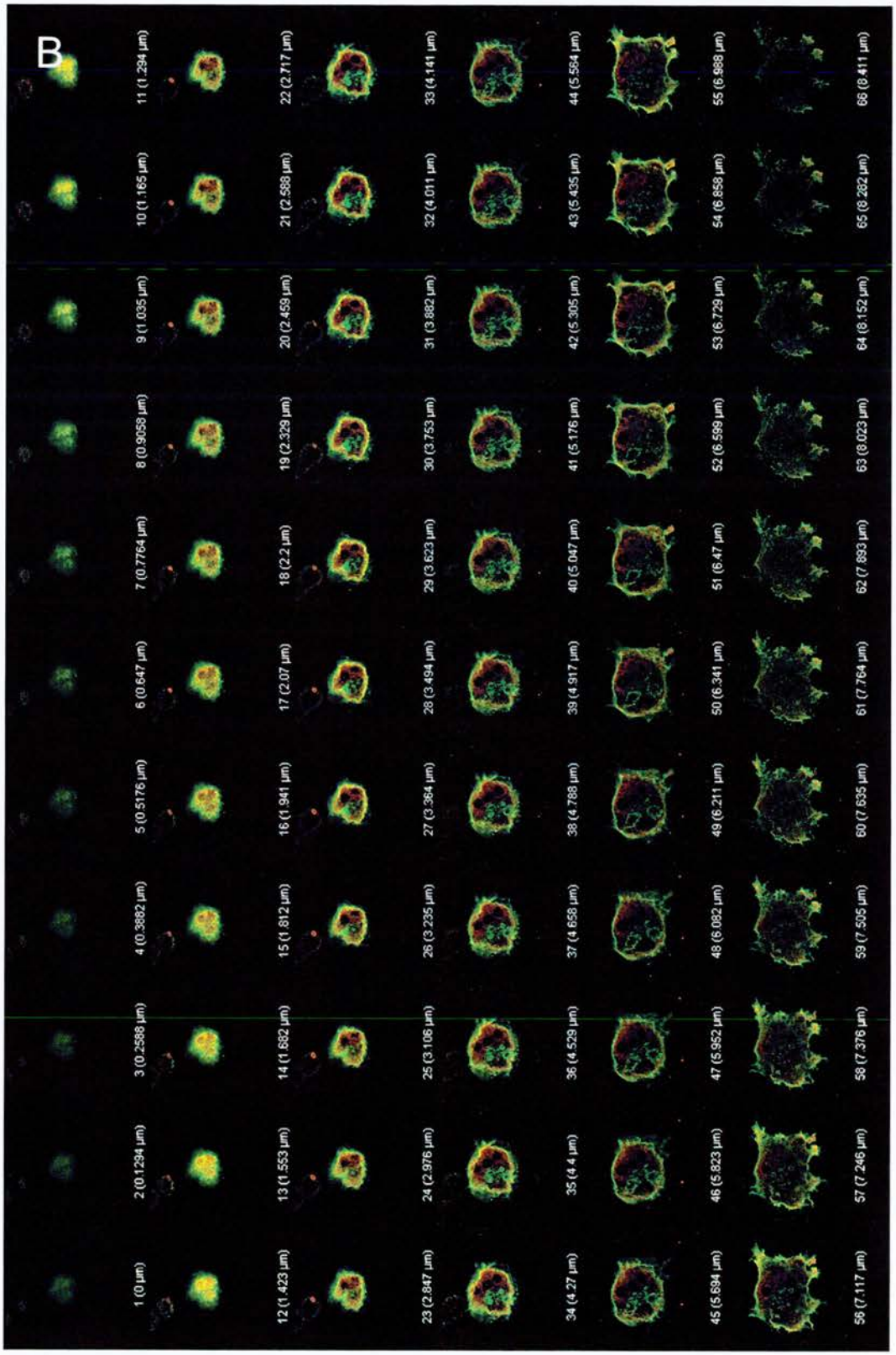
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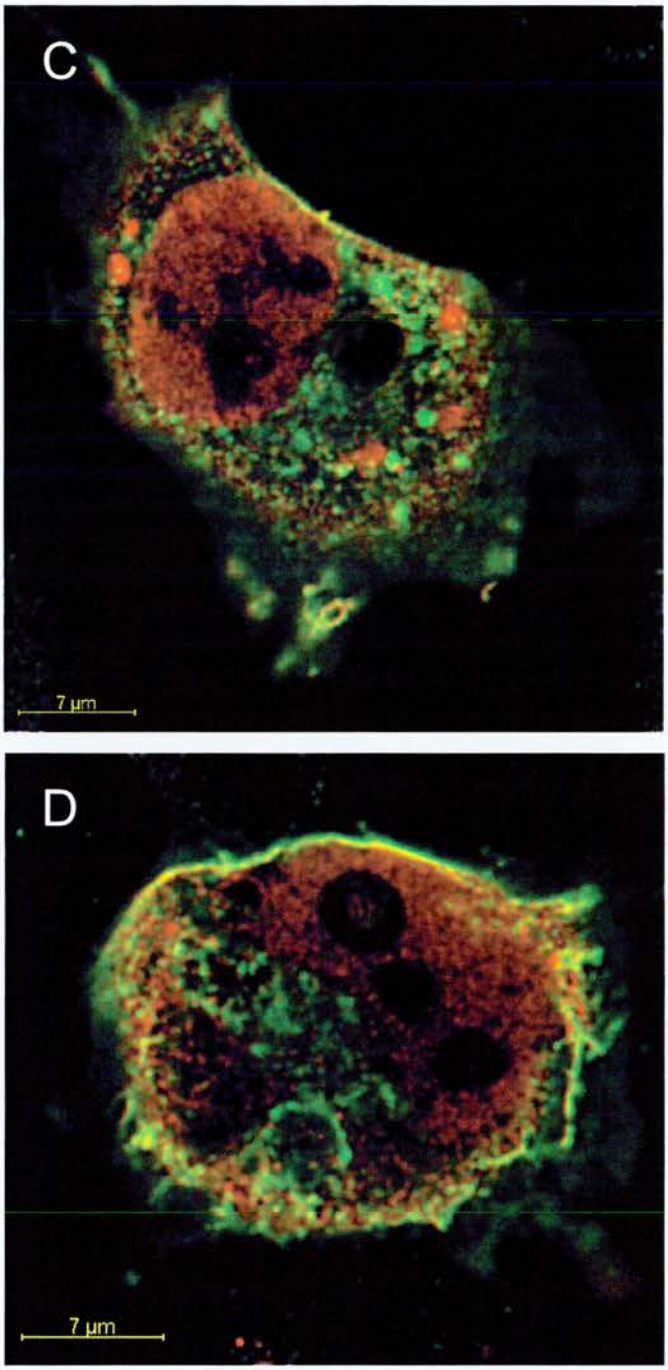
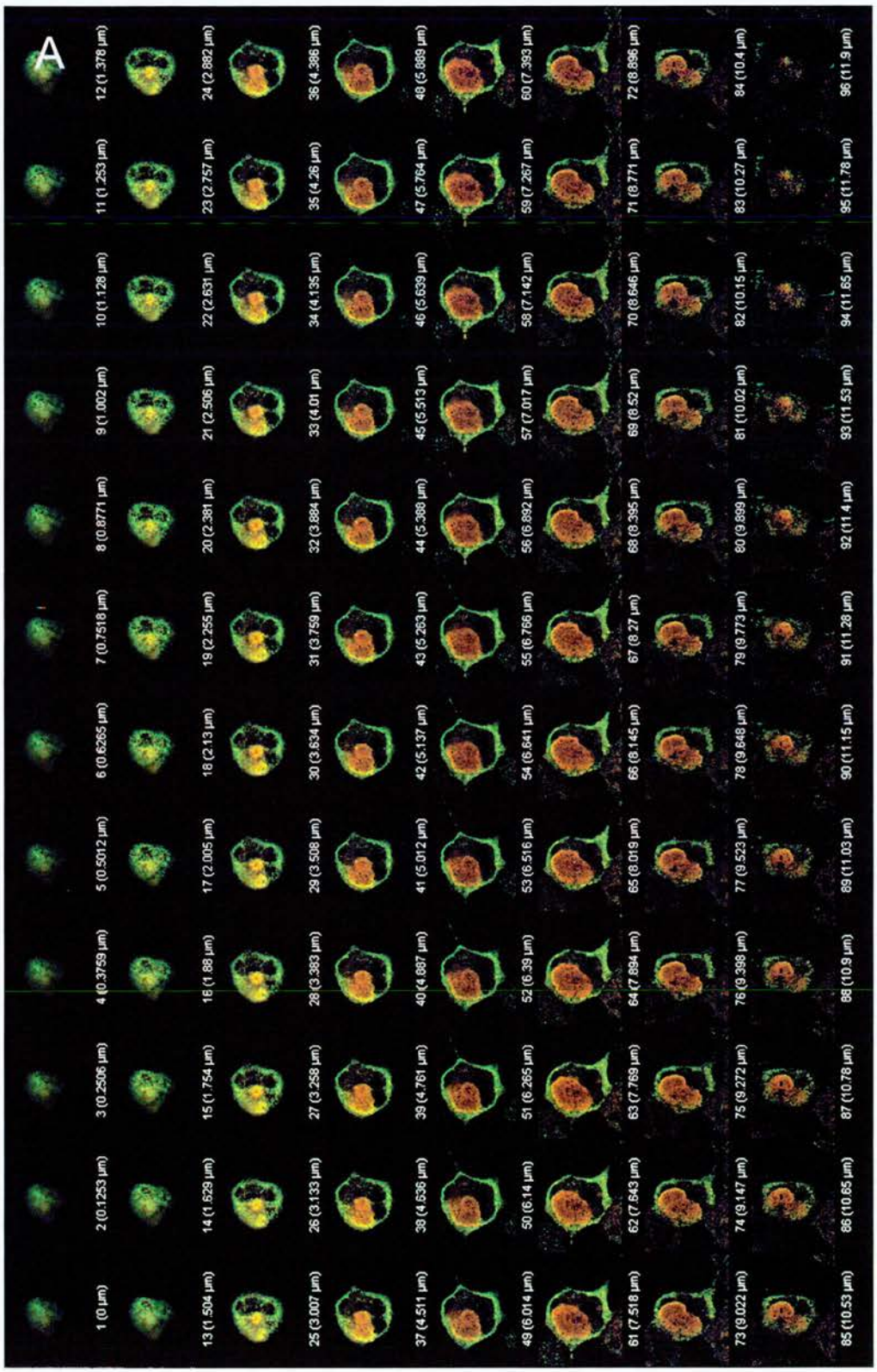


The effect of PKC stimulation on the localisation of PLD1 and 14-3-3ε.

The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD1 (green) and 14-3-3ε-myc (red).

Figure 5.12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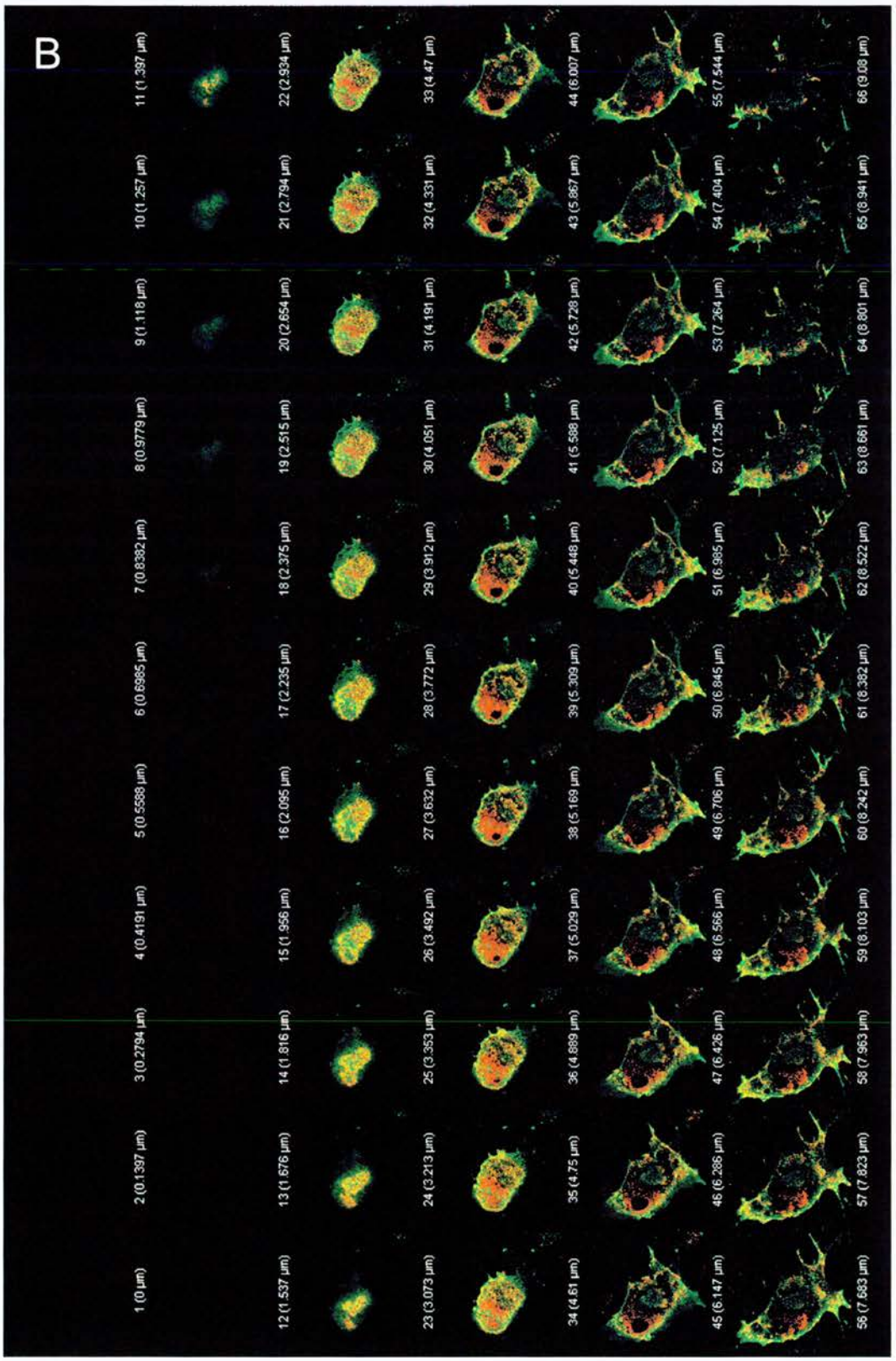
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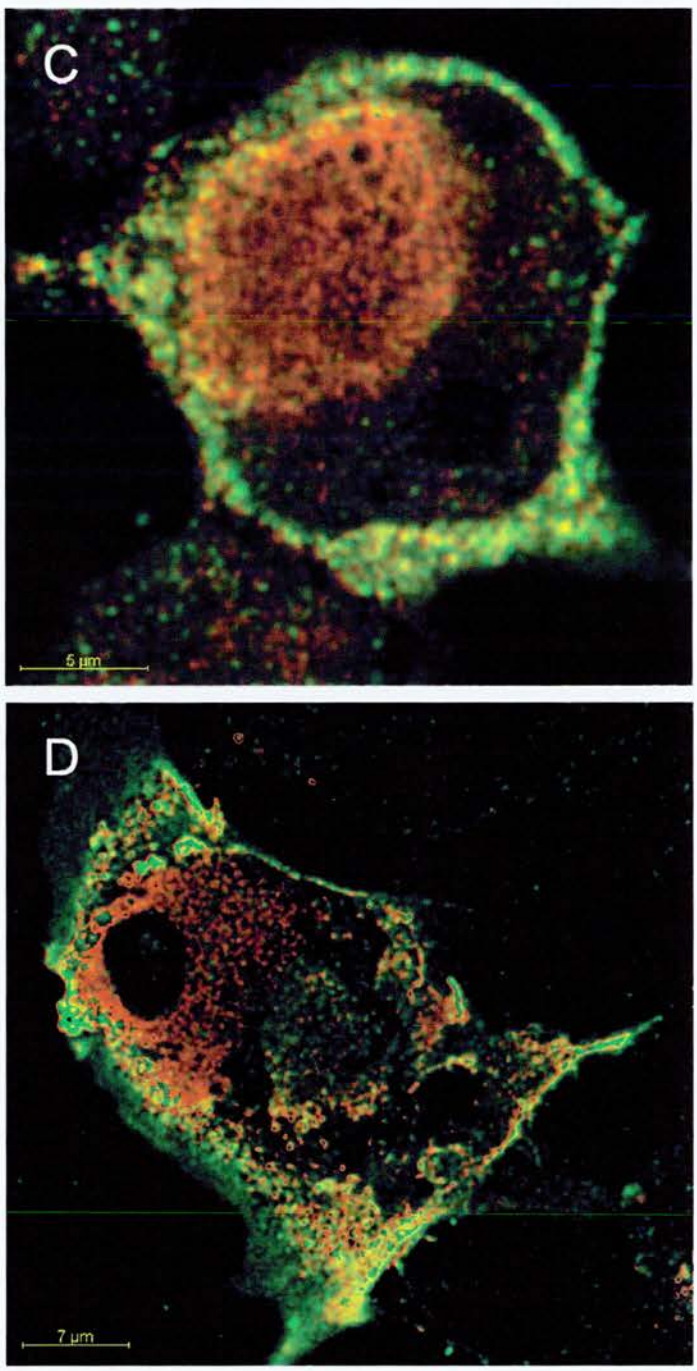


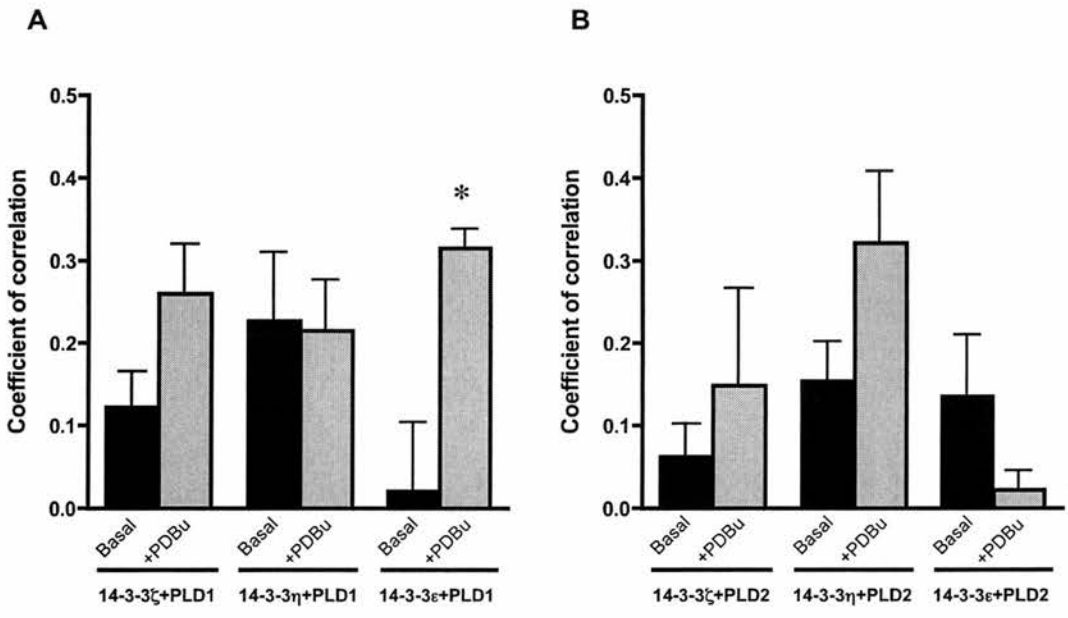
The effect of PKC stimulation on the localisation of PLD2 and 14-3-3ε.

The gallery (A) and mid-section (C) views of an unstimulated COS 7 cell compared with the gallery (B) and mid-section (D) views of a phorbol ester stimulated COS 7 cell (500 nM PDBu, 10 min) transiently transfected with HA-PLD2 (green) and 14-3-3ε-myc (red).

Taken together these values indicate that the change in colocalisation of the various 14-3-3 isoforms with PLD1 and PLD2 is isoform dependent. For PLD1, the colocalisation of 14-3-3η showed little difference between resting and stimulated COS 7 cells, whereas 14-3-3ε showed a significant increase ($n=3$, $p<0.05$; Unpaired student t-test) (Figure 5.13). The colocalisation of 14-3-3η with PLD2 appeared to increase with PKC stimulation, although not quite significantly ($p=0.054$; Unpaired Student t-test). The acquisition of further data to attain a higher *n* number may have enabled a more explicit interpretation, however a trend has emerged to suggest a distinct isoform specificity of interaction. It must be noted that the values for the colocalisation of the 14-3-3 isoforms with PLD are quite low indicating that the incidence of the two proteins colocalising, even though the interaction may be robust, is also low. This is not totally unexpected, as the sheer abundance of 14-3-3 interactions (with other binding partners) within the cell and the relatively low levels of PLD expression, may preclude the association from being particularly widespread. However, the evidence using GST-fusion and co-immunoprecipitation studies suggests that an interaction does take place *in vitro* and the imaging studies lend support to the idea of this interaction occurring *in vivo*.

Comparing the colocalisation results with the biochemical results yields a reasonable corroboration. The stimulation of PLD1 by PKC leads to its colocalisation with 14-3-3ε being significantly increased and leads to the co-immunoprecipitation of PLD1 with 14-3-3ε showing an increase. Furthermore, PKC stimulation does not readily affect PLD1 association with 14-3-3η, but PLD2 colocalisation (and potential association) may be increased. Preferential interactions of the different isoforms of 14-3-3 with the two PLD isozymes could potentially indicate a level of molecular control within the cellular system. It is currently unknown how 14-3-3 functions physiologically in this scheme, but there may be indications from other 14-3-3 interactions. For example, 14-3-3 dimers have been shown to be an important mediator in the coordination of two proteins together in an activation

Figure 5.13



The effect of PKC stimulation on the colocalisation of PLD and 14-3-3 isoforms.

The coefficient of correlation of voxels containing channel intensities for both PLD1 (A) or PLD2 (B) and different 14-3-3 isoforms is shown. Unstimulated COS 7 cells (black bars) ($n \geq 3$) and COS 7 cells that had been stimulated for 10 min with 500 nM of the phorbol ester phorbol 12,13-dibutyrate (grey bars) ($n \geq 3$) were imaged and the resulting change in correlation coefficient for the two channels recorded. The correlation coefficient is an indication of the two channels colocalisation - a value of 1 indicates all voxels of both channels are colocalised (perfect correlation), a value of 0 indicates no correlation and –1 indicates exclusive colocalisation (perfect inverse correlation) The stimulation of protein kinase C (PKC) by phorbol dibutyrate results in a change of location of 14-3-3 isoforms and a change in colocalisation of 14-3-3 and PLD. PKC stimulation significantly increases colocalisation of the 14-3-3ε isoform with PLD1 (*$p<0.05$; Unpaired Student t-test).

process, such as c-Raf with Ras or PKC (Sozeri *et al.*, 1992; Fantl *et al.*, 1994; Freed *et al.*, 1994). The 14-3-3 isoforms could potentially fulfil the same role in this system and preferentially facilitate direct coupling of PKC to PLD as an isoform-dependent functional linker molecule. Alternatively, 14-3-3 may bind in an isoform specific manner to PLD following activation (or subsequent phosphorylation) by PKC to modulate or facilitate the phosphorylation induced attenuation of PLD activity (Hu and Exton, 2003; Chen and Exton, 2004). Furthermore, there may also be the possibility that another protein (or proteins) may potentially be involved in the interaction.

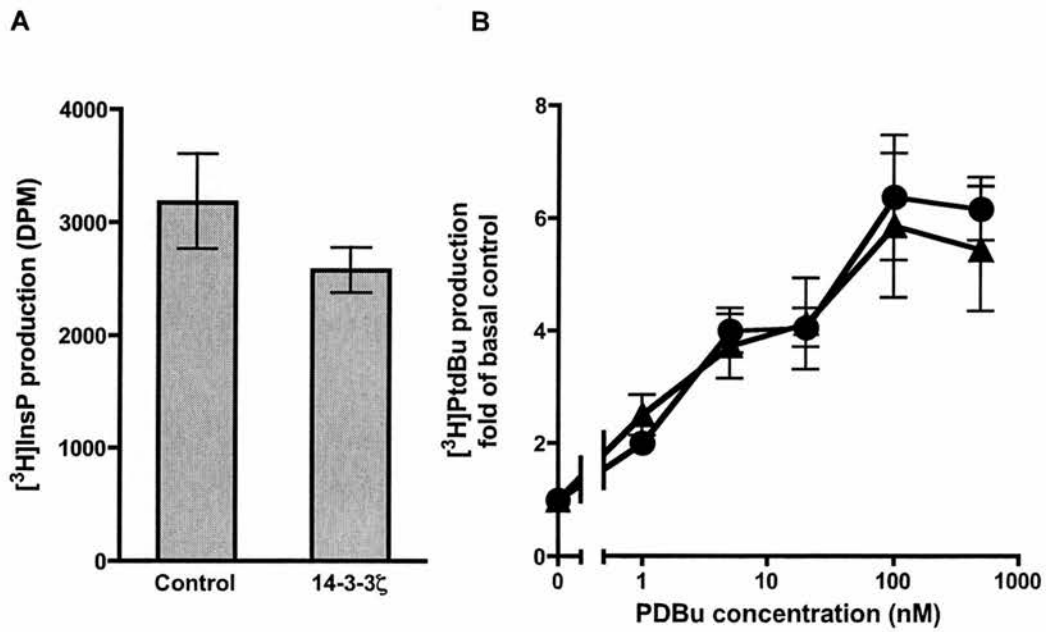
14-3-3 alters PKC mediated activation of PLD

To make a preliminary assessment of whether 14-3-3 association with PLD might be physiologically relevant *in vivo*, PLD assays in whole COS 7 cells, transiently transfected with 14-3-3 isoforms were performed. PLC assays were also performed as a control, to ensure that overexpression of 14-3-3 did not affect the signalling characteristics due to reduced cell viability. COS 7 cells, transiently transfected with empty control vector or 14-3-3ζ-myc were seeded into 24 well plates and were made quiescent 16 hours before the assay. Cells for PLC assays were radiolabelled for 16 hours with [^{3}H]inositol and cells for PLD assays were radiolabelled with [^{3}H]palmitate. The cells were stimulated with either 10 mM F^- and 50 μM Al^{3+} (AlF_4^-) to directly stimulate the heterotrimeric G protein and activate PLC, assaying the [^{3}H]inositol phosphate product as a control, or the cells were stimulated with different concentrations of phorbol dibutyrate (PDBu) to stimulate PKC and consequently activate PLD, assaying the [^{3}H]phosphatidylbutanol product. The responses of both PLC and PLD pathways to overexpression of 14-3-3ζ are shown in Figure 5.14. Overexpression of 14-3-3ζ caused a slight decrease in the response to PLC compared to the control vector, however this was not significant ($p>0.05$, Wilcoxon test; n=5) (Figure 5.14A). Furthermore, the signalling characteristics of PKC stimulated PLD were unaffected by the overexpression of 14-3-3ζ ($p>0.05$, Wilcoxon test; n=5) (Figure 5.14B).

The characteristics of 14-3-3ζ influences on receptor-independent PLC/PLD signalling are broadly similar to those of the agonist-stimulated muscarinic receptor responses found in Chapter 3. The small attenuation of the PLC response, although not significant, was consistent and indicates that 14-3-3ζ may negatively regulate the signalling of the heterotrimeric G protein to PLC. The close similarity of the PLD response to the control would indicate that 14-3-3ζ overexpression does not modulate this signalling pathway to a high degree. There could be a number of reasons for this; it may be because 14-3-3ζ does not interact with PLD to provide any kind of functional role. Alternatively, it may be that endogenous 14-3-3 can facilitate some aspect of the PKC-mediated stimulation of PLD and so overexpression of the 14-3-3ζ isoform does not alter it. Or it may demonstrate that interaction of the 14-3-3ζ with PLD may occur downstream of the PKC-mediated activation of PLD. It was therefore determined to observe other 14-3-3 isoforms to try and elucidate further whether there was isoform specificity involved in PKC-mediated PLD activity.

The signalling assays were repeated to observe the effect of overexpression of the 14-3-3η isoform. COS 7 cells were transiently transfected with empty control vector or myc-14-3-3η, made quiescent and radiolabelled with either [^{3}H]inositol or [^{3}H]palmitate 16 hours prior to the assay as before. The cells were then stimulated with AlF_4^- to activate the PLC pathway, or different concentrations of PDBu to activate the PKC-dependent PLD pathway, and the products of each were assayed for [^{3}H] incorporation (Figure 5.15). The overexpression of 14-3-3η did not significantly affect the PLC response in COS 7 cells compared to the control vector ($p>0.05$, Wilcoxon test; n=5). The PKC mediated PLD response however, was significantly impaired with the overexpression of the 14-3-3η isoform ($p<0.05$, Wilcoxon test; n=5) at PDBu concentrations of 100 nM.

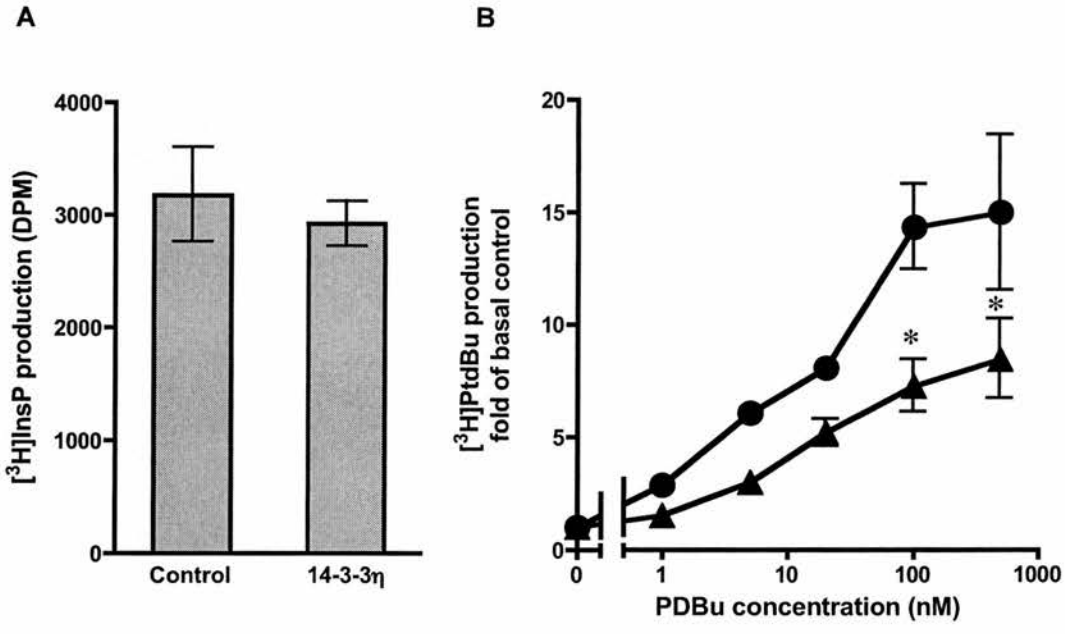
Figure 5.14



Overexpression of 14-3-3ζ does not affect PLC and PLD responses in COS 7 cells.

COS 7 cells, transiently transfected with empty control vector (●) or 14-3-3ζ-myc (▲) were stimulated for 20 minutes with either AlF_4^- (to directly activate the heterotrimeric G-protein coupled PLC pathway; A) or various concentrations of the PKC activator phorbol dibutyrate (to activate PLD; B). Although slightly reduced from those in the presence of control vector, the PLC responses were not significantly affected by the expression of 14-3-3ζ ($p>0.05$, Wilcoxon test; n=5). Furthermore, the phorbol ester-induced PLD responses were not significantly different between control and 14-3-3ζ expressing cells ($p>0.05$, Wilcoxon test; n=5).

Figure 5.15



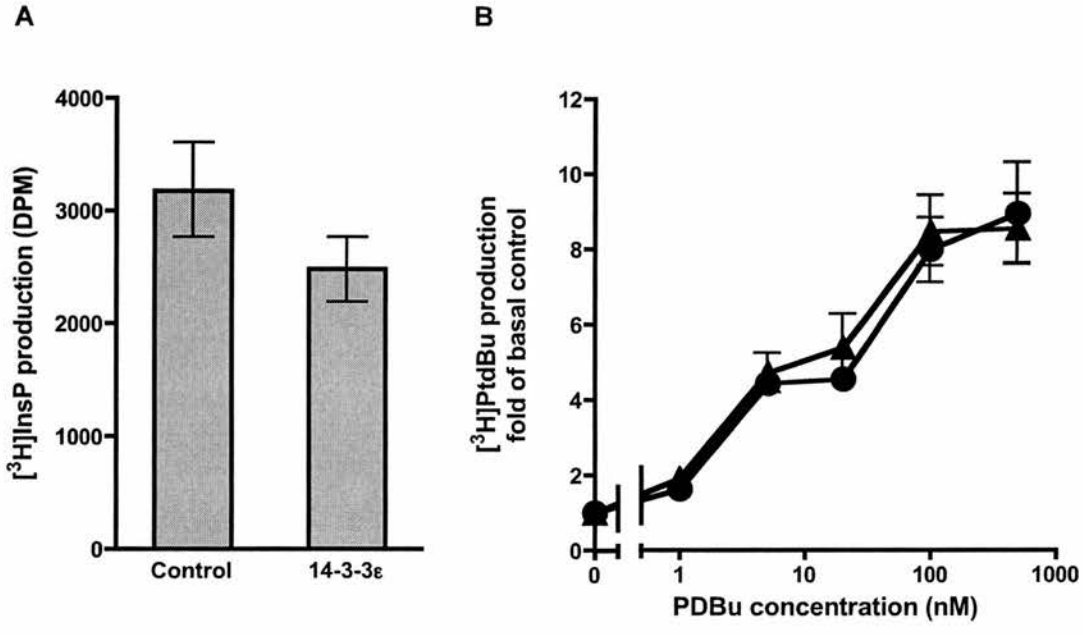
Overexpression of 14-3-3η affects PLD but not PLC responses in COS 7 cells.

COS 7 cells, transiently transfected with empty control vector (●) or myc-14-3-3η (▲) were stimulated for 20 minutes with either AlF_4^- (to directly activate the heterotrimeric G-protein coupled PLC pathway; A) or various concentrations of the PKC activator phorbol dibutyrate (to activate PLD; B). The response to AlF_4^- stimulation of PLC was not affected by overexpression of 14-3-3η ($p>0.05$, Wilcoxon test; n=5), however the PKC mediated PLD response was significantly attenuated by overexpression of the 14-3-3η isoform (*$p<0.05$, Wilcoxon test; n=5).

These data indicate that the isoform specificity of the 14-3-3 interaction with PLD extends to a functional distinction. The attenuation of the PLD response with the overexpression of 14-3-3η suggests that 14-3-3 proteins do interact with PLD (or potentially PKC) in a way that can modulate the activity of PLD in an isoform dependent manner. One reason for this difference may be that the myc tag of 14-3-3η is at the amino-terminus of the protein compared to the carboxy-terminus on the 14-3-3ζ construct and this may impede some aspect of the interaction that is necessary *in vivo* (but perhaps not the interaction *in vitro*). However, this is unlikely, as the folding characteristics of the 14-3-3 dimer, coupled with other experimental results, indicate that the carboxy-terminal myc-tag should not interfere with 14-3-3 target association. Alternatively, the possible isoform specificity of the 14-3-3η interaction with PLD2 compared to PLD1 may cause the functional differences observed. It is known that in COS 7 cells, the majority of M_3 receptor-stimulated PLD activation is due to the PLD1 isozyme (Mitchell *et al.*, 2003) and it has been proposed that the basal activity of PLD2 is higher (Sung *et al.*, 1999). The overexpression of the 14-3-3η isoform, which may preferentially couple to PLD2 rather than PLD1, might possibly result in the competitive inhibition of PLD1 activation by PKC. This may be due to the 14-3-3η coordinating the 'incorrect' PLD isozyme to PKC for activation (if the 14-3-3 proteins generally perform such a role) or it may be due to some modulation of PLD post-activation. Further information about the relative PLD isozyme activation by PKC (for example mutagenesis studies) would be valuable in this cellular system.

The signalling assays were repeated for the 14-3-3ε isoform in COS 7 cells. Empty control vector or 14-3-3ε-transfected COS 7 cells were assayed for the production of either [^{3}H]inositol phosphate (in response to AlF_4^- mediated PLC stimulation) or [^{3}H]phosphatidylbutanol (in response to PDBu-mediated, PKC-activated PLD stimulation) (Figure 5.16). The PLC response to AlF_4^- in cells overexpressing 14-3-3ε was moderately attenuated compared to the control cells, although this was not statistically significant

Figure 5.16



Overexpression of 14-3-3ε does not affect PLC or PLD responses in COS 7 cells.

COS 7 cells, transiently transfected with empty control vector (●) or 14-3-3ε-myc (▲) were stimulated for 20 minutes with either AlF_4^- (to directly activate the heterotrimeric G-protein coupled PLC pathway; A) or various concentrations of the PKC activator phorbol dibutyrate (to activate PLD; B). Although reduced from control vector expression, the PLC responses were not significantly affected by the overexpression of 14-3-3ε ($p>0.05$, Wilcoxon test; n=5). Furthermore, the signalling characteristics of PLD were not significantly different between control and 14-3-3ε expressing cells ($p>0.05$, Wilcoxon test; n=5).

($p>0.05$, n=5). The PLD response to PDBu was unaffected by the overexpression of the 14-3-3ε isoform, similar to the lack of effect of the 14-3-3ζ isoform. It may be of relevance that the myc-tag on 14-3-3ε construct is at the carboxy-terminal domain, as for 14-3-3ζ, which also failed to affect functional PLD responses to PDBu (as above). The characteristics of the signalling responses with the overexpression of 14-3-3ε were thus generally quite similar to those of the 14-3-3ζ isoform and this suggests that the isoform specificity may function as a potential switch for facilitating the activation of one pathway (PLD) over another (PLC).

Summary

In this chapter, the interaction of 14-3-3 with PLD has been investigated as a result of the novel observation that PLD isozymes contain consensus 14-3-3 interaction sequences, based on the well characterised c-Raf RSxpSxP motif. Scansite analysis of the PLD sequences reveals that the potential 14-3-3 recognition motif in PLD1, RSLSYP (712-717) is a high stringency mode-1 phosphoserine based recognition sequence and that PLD2 has two lower stringency mode-1 14-3-3 recognition motifs, RLLTMS (172-177) or KTPTYP (573-579) (Obenauer *et al.*, 2003). Furthermore, it has been found that isoforms of 14-3-3 may interact with PLD isozymes in an isoform dependent manner *in vitro*. In addition, the 14-3-3 isoforms co-immunoprecipitate with PLD1 and PLD2 with apparently different affinities and the co-immunoprecipitation levels of 14-3-3 with PLD may potentially be modulated by PKC activation. In addition, the intracellular colocalisation of the 14-3-3 isoforms with PLDs has been investigated and it has been demonstrated that PKC stimulation appears to alter the colocalisation characteristics of the proteins, again in an isoform dependent way. Moreover, the overexpression of 14-3-3 isoforms has been shown to differentially modulate the signalling characteristics of PKC-stimulated PLD responses in whole COS 7 cells, with the unexpected finding that 14-3-3η significantly attenuates the PKC-mediated PLD response in COS 7 cells.

These results suggest that 14-3-3 proteins do play an important and isoform dependent functional role in the signalling of PLD *in vivo*, however the precise nature of this role remains uncertain. Site-directed mutagenesis of the residues within PLD, thought to be involved in the interaction with 14-3-3 (the putative consensus sequences), would be very useful to determine whether the interaction took place at this location. Indeed, these mutants would also yield valuable information with regard to the functional role of the association in the PLD signalling response and whether 14-3-3 acted to facilitate the PKC and PLD interaction (by coordinating them together as an adapter) or whether 14-3-3 acted downstream of PLD activation (for example to facilitate the later PKC-mediated phosphorylation and inactivation of PLD). Furthermore, determining the kinase that acts to phosphorylate the potential 14-3-3 consensus sites on PLD would be very valuable, and the possibility that PKC may be the kinase that phosphorylates both PLD1 and PLD2 on these sites, to later recruit 14-3-3 following PLD activation, remains feasible as they are all medium stringency sites for PKC phosphorylation (Obenauer *et al.*, 2003). Other kinases that may potentially be responsible are Akt kinase for the domain on PLD1 and calmodulin dependent kinase 2 for the domain on PLD2 (Obenauer *et al.*, 2003). Moreover, the recruitment of 14-3-3 may also perform some other function, such as facilitating the activation of alternative intracellular signalling pathways or the facilitation of a PLD dependent trafficking mechanism.

Additionally, it would be desirable to determine whether 14-3-3 proteins may act at multiple stages of the signalling pathway, by investigating whether RGS proteins (by using catalytically inactive RGS mutants) or PKC (by using specific inhibitors) were critical in the 14-3-3 modulation of any whole cell PLD signalling response. The investigation of other 14-3-3 isoforms (for example the homodimeric 14-3-3γ) and their effect on the signalling of PLD would also be of interest. Moreover, the use of live cell imaging and FRET with fluorescently labelled PLD and 14-3-3 would be able to yield much more information with respect to the nature of any PLD/14-3-3 interaction *in vivo*. It is clear that 14-3-3 may play

an important role in GPCR signalling and that of related downstream pathways, such as PKC activation, however much work still needs to be performed to fully elucidate what that role may be.

Chapter 6:

Conclusions and Discussion

Conclusions and discussion

The delineation of signalling mechanisms is potentially an important route for developing novel therapeutic targets. Traditionally, pharmacological agents have been developed that act on cell surface receptors to modulate the intracellular environment. However, as the complexity of signalling pathways becomes more and more apparent, it may be necessary to investigate other, more specific, potential targets for drug design. In this study, the nature of a novel involvement of 14-3-3 proteins in the phospholipid signalling mechanism of the M_3 muscarinic receptor has been investigated. This may serve as a model for the organisation of other G protein-coupled receptor mechanisms. It has been shown in Chapter 3 of this study that the interaction of 14-3-3 isoforms with the M_3 muscarinic receptor is similar to that of the previous findings made by Prezeau and colleagues in the class A α_2-adrenergic GPCR and the findings made by Couve and colleagues in the class C metabotropic $GABA_B$ GPCR (Prezeau *et al.*, 1999; Couve *et al.*, 2001). In those systems, the association of 14-3-3 with the receptors was postulated to be providing a potential scaffolding or receptor localisation function. However, the specific co-immunoprecipitation of the receptors with the 14-3-3 proteins was not demonstrated in those studies, suggesting that the affinity of the 14-3-3 interaction with the M_3 receptor, in this system, is potentially higher than with those other GPCRs (Prezeau *et al.*, 1999; Couve *et al.*, 2001). Furthermore, 14-3-3 proteins have been shown to associate with other plasma membrane-associated proteins such as potassium channels to provide a functional role in mediating the release of correctly assembled multimeric channels from the endoplasmic reticulum (ER) or in targeting these proteins correctly to the plasma membrane (Rajan *et al.*, 2002; Yuan *et al.*, 2003). The association of 14-3-3 with the M_3 receptor is consistent with this type of role and it may be the case that 14-3-3 can facilitate M_3 receptor trafficking from the trans-Golgi network, or 14-3-3 may contribute to coordinating the insertion of the receptor into the plasma membrane. This role is of interest in view of our further evidence, as demonstrated in Chapter 5, for interactions

of PLD with 14-3-3 and the M_3 receptor, since the ARF-dependent PLD activity in the Golgi apparatus is thought to be important for vesicle budding from the trans-Golgi network (Ktistakis *et al.*, 1995; Ktistakis *et al.*, 1996; Chen *et al.*, 1997). Moreover, homologues of 14-3-3 proteins in the budding yeast *Saccharomyces cerevisiae*, BMH1 and BMH2, have also been shown to be important in vesicular trafficking, where they can rescue Δchc^- non-viable strains, which are gene deletion mutants (knockouts) of the clathrin heavy chain proteins (a coat protein involved in vesicular transport) (Gelperin *et al.*, 1995). 14-3-3 proteins have also been shown to associate with phospholipid membranes in an isoform-dependent manner (Roth *et al.*, 1994; Jones *et al.*, 1995). In view of the role of clathrin in GPCR endocytosis (Zhang *et al.*, 1996; Laporte *et al.*, 1999; Oakley *et al.*, 1999; Pierce *et al.*, 2000) the association of 14-3-3 with the M_3 receptor indicates that 14-3-3, in addition to providing a scaffolding role, may facilitate some type of exocytic/endocytic protein assembly, for insertion into, or sequestration away from, the plasma membrane. It is known that many GPCRs, including the M_3 receptor, use a clathrin (and dynamin)-dependent process for internalisation and this endocytic process may also involve PLD activity (Vogler *et al.*, 1999; Koch *et al.*, 2003; Johnson *et al.*, 2004). The potential for a role of 14-3-3 in such mechanisms has not yet been directly investigated. However, there are thought to be several atypical routes for GPCR endocytosis that remain mechanistically undefined (Claing *et al.*, 2000) and which may potentially involve 14-3-3 and PLD.

The demonstration in Chapter 4 that phospholipase D isozymes can interact with the M_3 muscarinic receptor is consistent with a potential role of PLD activation in mediating endocytosis of GPCRs, as proposed by other groups (Koch *et al.*, 2003; Du *et al.*, 2004; Koch *et al.*, 2004). Evidence was provided to suggest that the endocytosis and recycling of the μ-opioid G protein-coupled receptor was dependent upon interaction with PLD2, but not PLD1 (Koch *et al.*, 2003). This may indicate that the efficient sequestration of at least some of the receptors from the cell surface and perhaps the subsequent recycling of these

receptors back to the plasma membrane could be dependent on PLD activity (Koch *et al.*, 2004). These results were supported by another group, who observed that the internalisation of the angiotensin II (AT_{1A}) receptor was also dependent upon PLD2 activity, although any direct interaction between PLD2 and the AT_{1A} receptor was not addressed (Du *et al.*, 2004). In the case of the angiotensin II receptor, it has been shown that PLD2 is the isozyme that is predominantly activated upon agonist stimulation in A10 cells (Shome *et al.*, 2000), indicating that the preferentially coupled PLD isozyme may also mediate the internalisation of the receptor. In other experiments performed by our group, we have shown that PLD1 is the isozyme that is preferentially activated by the M_3 receptor in COS 7 cells (Mitchell *et al.*, 2003) and determined that catalytically inactive mutants of both PLD1 and PLD2 isozymes, as well as the PLD inhibitor calphostin C, can inhibit agonist mediated M_3 receptor internalisation (Johnson *et al.*, 2004). This therefore suggests that PLD1 is likely to be able to mediate internalisation of the M_3 receptor in this system. Moreover, receptor-mediated PLD activity has been demonstrated to be important for internalisation of other receptor classes, for example the epidermal growth factor (EGF) receptor tyrosine kinase (Shen *et al.*, 2001). PLD2 was shown to associate with the EGF receptor in a ligand-independent manner (similarly to the findings within Chapter 4) and PLD1 was determined to be the main mediator of internalisation *via* the activation of PKC (Slaaby *et al.*, 1998; Shen *et al.*, 2001). Our findings indicate that either PLD1 or PLD2 may mediate M_3 receptor internalisation, however the implication that PLD1 is the preferred isozyme may be supported by the evidence for a level of dissociation and subsequent re-association of PLD1 from the M_3 receptor following agonist stimulation (Figures 4.6-4.7). The product of PLD activity, phosphatidic acid, may be the bioactive species necessary for facilitating part of the endocytic mechanism. PA has been shown to facilitate the binding of the GTPase dynamin to lipid membranes to assist the formation of endocytic vesicles (Burger *et al.*, 2000). The precise mode of action is uncertain, however PA (with a small, highly polar head group)

may alter the physical organisation of the plasma membrane to destabilise the interior lipid:cytosolic interface and allow efficient vesicle budding (Andresen *et al.*, 2002). Moreover, PA has been implicated in facilitating the recruitment of the AP-2 adapter complex and clathrin coats to lysosomal membranes, which is dependent on PIP_2 synthesis arising from the activation of phosphatidylinositol 4-phosphate 5-kinase by phosphatidic acid (Arneson *et al.*, 1999). The activation of PLD by the M_3 receptor and subsequent production of PA may provide a positive feedback mechanism for the necessary *de novo* synthesis of PIP_2 on the plasma membrane, and facilitate the sequestration of the receptor away from the cell surface by modification of the lipid membrane or initiating the assembly of endocytic machinery.

Evidence has been provided that EGF-induced activation of the MAPK pathway was dependent on the PLD-mediated endocytosis of the EGF receptor (Shen *et al.*, 2001). Moreover, the co-activation of the MAPK cascade by some GPCRs has been shown to be dependent upon clathrin-mediated endocytosis, whilst in other GPCRs it is not (Pierce *et al.*, 2000). However, several studies have revealed that inhibitors of the endocytic pathway can act to inhibit co-activation of MAPK, regardless of whether or not the GPCR undergoes endocytosis, as the co-activation of MAPK by agonist stimulation of the α_{2A}-adrenergic receptor (which is not endocytosed) could be prevented by monodansylcadaverine (a blocker of an important endocytic protein – transglutaminase), a mutant dynamin and a mutant beta-arrestin (Pierce *et al.*, 2000). The M_3 muscarinic receptor has been shown to activate the MAP kinase pathway upon receptor stimulation and various reports suggest this may or may not be dependent on internalisation (Bertram *et al.*, 1998; Budd *et al.*, 2000; Budd *et al.*, 2001). Additionally, phosphatidic acid, the product of PLD activity, has been shown to be important for full activation of the MAPK pathway (Rizzo *et al.*, 1999; Andresen *et al.*, 2002; Ghosh *et al.*, 2003) and is produced concomitantly with M_3 receptor activation and internalisation. It is quite possible that the functional mechanisms arising

from agonist stimulation, involving the activation of PLD, the production of phosphatidic acid and the subsequent endocytosis of the M_3 receptor, may facilitate the activation of a signal crosstalk system that contributes to cellular MAPK pathway activation.

Furthermore, it has been previously demonstrated that 14-3-3 proteins interact with c-Raf in the activation of the MAP kinase pathway, either by Ras or by PKCα (Kolch *et al.*, 1993; Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994). 14-3-3 has been shown to have an important trafficking and regulating role in this pathway, as it facilitates the translocation and regulation of the cytosolic c-Raf to the membrane for activation (and has been implicated in the activation mechanism itself) (Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994). Therefore the (possibly PLD dependent) co-activation of MAPK by the M_3 receptor may be facilitated by the presence of 14-3-3, which may perform some functional adapter, trafficking or scaffolding role. The 14-3-3 dimer may act to coordinate two proteins together for activation, or it may be recruited by regulatory modulation of another protein to facilitate the transport of that protein to another cellular compartment. The interaction of 14-3-3 with the PLD isozymes in an isoform-dependent manner, as presented in Chapter 5, is consistent with this type of role. The potential 14-3-3 interaction motifs in PLD2 (KTPTYP or RLLTMS) are not as optimal for 14-3-3 interaction as that of PLD1 (RSLSYP) (Obenauer *et al.*, 2003). The divergent nature of the site may indicate that some isoforms of 14-3-3 are more particularly suited to interacting with PLD2 than others (compared to the similar affinities of interaction of 14-3-3 with the more optimal, putative PLD1 motif). In this case, a candidate for specificity conferred by the site would be the 14-3-3η isoform, as inferred by the results in Chapter 5. The interaction of PLD2 with 14-3-3η appears to have a potentially higher affinity than that between PLD2 and the other 14-3-3 isoforms. This contrasts with PLD1, which interacts with 14-3-3ζ and 14-3-3η with an apparently similar affinity (although they are not necessarily colocalised to the same extent). The isoform specificity of 14-3-3 interaction

with PLD may provide functional regulation of PLD localisation or activity and hence influence downstream effects. Such a level of functional regulation, potentially provided by the isoform specificity of interaction, is supported by the results of the whole cell PLD signalling responses of Chapter 5. When the isoforms of 14-3-3 were overexpressed in COS 7 cells, the 14-3-3ζ and 14-3-3ε isoforms did not markedly affect PKC-activated PLD responses, compared to 14-3-3η, which significantly attenuated the PLD response (Figure 5.15). Whilst the modulation of the PKC-mediated PLD response with 14-3-3η may be attributed to conformational disruption due to the site of the tag (and this itself implies a functional role for 14-3-3), an alternative reason for the effect of 14-3-3η on PLD responses could be that overexpression of 14-3-3η may functionally inhibit the normal physiological PKC-mediated activation of PLD (which may involve 14-3-3ζ or 14-3-3ε, as overexpression of these did not affect PKC-mediated PLD responses). This may be because 14-3-3η might preferentially interact with PLD2 compared to PLD1 (and PLD2 does not mediate the same functional role as PLD1 in this system), or it may be that 14-3-3η has a distinctly isoform-specific role that may facilitate inhibition of the PLD response. The isoform specificity of the 14-3-3 association with PLD, coupled with the modulation of interaction observed upon PKC stimulation, would suggest that 14-3-3 may mediate a functional role during or after PLD activation rather than prior to activation. It has been characterised previously that 14-3-3 and PKC isoforms directly interact (Meller *et al.*, 1996; Matto-Yelin *et al.*, 1997). As PKC and PLD1 also associate directly *in vivo* (Singer *et al.*, 1996; Zhang *et al.*, 1999), 14-3-3 may potentially act as an adapter or to scaffold and stabilise the PKC/PLD interaction. The association of PKCα with both PLD1 and PLD2 isozymes is known to be increased with phorbol ester stimulation of PKC (Lee *et al.*, 1997; Sung *et al.*, 1999; Chen and Exton, 2004). Furthermore, preliminary data suggest that the interaction of 14-3-3ε with PLD1 and perhaps also the interaction of 14-3-3η with PLD2 may increase with PKC stimulation (Figures 5.4-5.6). One speculative role that 14-3-3

might perform, may be to bind to PLD following activation by PKC and to facilitate inhibition of the enzyme by binding to serine or threonine residues (phosphorylated by PKC or some other unknown kinase) in the RSxpSxP-type 14-3-3 binding motif on PLD, to alter some regulatory mechanism or to sequester PLD to another cellular compartment. Whilst a major component of the M_3 receptor-stimulated, ARF-mediated, PLD signalling pathway is PLD1-dependent in COS 7 cells (Mitchell *et al.*, 2003), the PKC-stimulated PLD activity may be potentially due to either of the isozymes. It has been proposed that PLD2 has a higher basal activity *in vivo* and is not as sensitive to activation by the other intracellular activators PKC, ARF and Rho (Jones *et al.*, 1999). However, other groups have indicated that PKC activates PLD2 with high potency (Chen and Exton, 2004). Determining the PLD isozyme contribution from PKC-mediated PLD activity could indicate whether PLD2 activity represents an important component of the PKC-mediated PLD activity in COS 7 cells (and therefore whether it is more sensitive to 14-3-3 isoform overexpression). The 14-3-3 dimer may then bind to PLD to potentially sequester PLD to another subcellular compartment or 14-3-3 may bind to PLD to regulate or physically occlude an important site on the enzyme (for example the catalytic HKD motif) in a similar way to the function that 14-3-3 performs with the RGS proteins.

The interaction of 14-3-3 with the regulator of G-protein signalling (RGS) proteins (Benzing *et al.*, 2000) has implicated 14-3-3 as providing a functional molecular switch for the modulation of G-protein families and their downstream effectors. This study has shown that overexpression of wild type and mutant 14-3-3 isoforms can functionally affect the activation of M_3 receptor-dependent PLC (Chapter 3) and PKC-dependent PLD (Chapter 5) pathways in COS 7 cells. The significant attenuation of the M_3 muscarinic receptor-activated PLC responses by expression of wild type 14-3-3ε (Figure 3.9) and the less marked, but similar trend with overexpression of 14-3-3ζ (Figure 3.8) (but not by overexpression of the 14-3-3 mutants) indicates that there may be a role for 14-3-3 isoform

regulation of the PLC pathway. It has previously been shown that RGS proteins can modulate heterotrimeric G proteins to preferentially couple the active receptor to different intracellular effectors (Rumenapp *et al.*, 2001). The results from the functional signalling experiments in this study could be considered to be consistent with a potential role for 14-3-3 in negatively regulating specific RGS subtype activity, as proposed by Benzing and colleagues (Benzing *et al.*, 2000). The modulation of M_3 receptor-dependent (as shown in Figures 3.8-3.9), but not AlF_4^- dependent (as shown in Figures 5.14-5.16), PLC responses by overexpression of wild-type 14-3-3 isoforms would be consistent with the proposed 'molecular switch' mechanism of negative regulation of RGS proteins by 14-3-3 proteins. If the PLC response to AlF_4^- stimulation potentially involves additional heterotrimeric G proteins (and hence potentially additional RGS proteins) to those involved in mediating the M_3 receptor-induced PLC pathway, the expression of wild-type 14-3-3 and the resulting modulation of the PLC signalling responses may specifically affect a subgroup of RGS proteins; therefore only marginally attenuating a response to AlF_4^- in contrast to the greater effect on M_3 receptor responses. However, as the 14-3-3 proteins have only been demonstrated to interact with RGS3 and RGS7 but not RGS4 (which is an RGS protein demonstrated to act on Gq G proteins), any potential 14-3-3 isoform-specific effects may not be attributable to this mechanism (Rumenapp *et al.*, 2001; Benzing *et al.*, 2002). Further investigations would be necessary to determine more precisely the nature of 14-3-3 modulation of the M_3 receptor-mediated signalling responses and any involvement of further RGS subtypes.

Originally it was hypothesised that 14-3-3 may act as a functional linker protein to potentially coordinate PLD to the M_3 receptor for activation. The functional results of the overexpression of the reduced affinity 14-3-3ζ (T233D 14-3-3ζ) and dimerisation deficient 14-3-3ε (Δ^-26 14-3-3ε) mutants provided in Chapter 3 would seem to imply that this is not the case, as they would be expected to modulate the M_3 receptor-mediated PLD response if

14-3-3 coordinated an M_3 receptor:PLD interaction. However, other isoforms that have not been investigated may have an additional functional affect on PLD responses *in vivo*. It has been shown in Chapter 5 that the interaction between 14-3-3 and PLD is also modulated by PKC stimulation and the isoform of 14-3-3 that is involved. This suggests that 14-3-3 may possibly function to facilitate the association, activation or inactivation of PLD by PKC. In addition, the modulation by 14-3-3 proteins of the signalling by the M_3 receptor to different effector pathways, indicates that there may potentially be two distinct functional roles for 14-3-3 in the regulation of intracellular effectors by the M_3 receptor. The first role supports the findings that 14-3-3 may regulate RGS activity (and may be consistent with the association of 14-3-3 with the M_3 receptor, as found in Chapter 3), indicating that 14-3-3 may regulate heterotrimeric G protein signalling to PLC (and possibly PLD) pathways. The second role is suggested by evidence for the involvement of PLD in the internalisation of the activated M_3 receptor and the 14-3-3 isoform specific and PKC-modulated association with PLD (as demonstrated in Chapters 4 and 5): This may indicate an involvement of 14-3-3 with the potential co-activation of alternative intracellular signalling pathways following cellular stimulation, or indeed with facilitating the potential PLD-dependent endocytic mechanism of M_3 receptor internalisation as demonstrated in Chapter 4.

Concluding remarks

The functional and isoform specific interaction of the 14-3-3 proteins with the M_3 muscarinic receptor and PLD isozymes has indicated an additional level of intracellular regulation of GPCR signalling mechanisms that may be physiologically relevant.
The ligand recognition site for GPCRs has been one of the main targets for drug action - to prevent or mimic the cellular influence of GPCR activation in a wide variety of physiological systems. However, the activation of novel intracellular signalling mechanisms (and the proteins that may potentially be a part of these mechanisms) may have important implications for further drug therapies. Therefore further elucidating the intracellular consequences of receptor activation is of major importance. This study has shown that the combined techniques of biochemical analysis (GST-fusion and co-immunoprecipitation studies) combined with confocal microscopy and functional pharmacological signalling assays have been an important and powerful approach to determining these novel intracellular interactions.

The involvement of the 14-3-3 proteins and PLD in the signalling by the M_3 receptor has been demonstrated to be functionally important, however there remains a dearth of information on the specific coupling mechanisms that would be valuable for future investigation. Future studies may benefit from the use of fluorescence-tagged (for example, enhanced cyan or yellow fluorescent protein; eCFP or eYFP) 14-3-3 and PLD isozymes in order to determine whether they interact together in fixed or living cells with the use of fluorescent resonance energy transfer analysis. This technique can identify whether the two proteins are in extremely close proximity (a distance of less than 100 Å) by measuring the activation (energy emission) of an acceptor fluorophore by a donor fluorophore without the emission of a photon. Further studies may also involve the collection of more signalling data on the other 14-3-3 isoforms and mutants of those already investigated. In addition, site-directed mutagenesis of the serine or threonine residues that are expected to be key elements

within the RSxpSxP type 14-3-3 consensus domains on the PLD isozymes would be extremely informative. These mutants could be expressed in whole cells to determine more specifically the functional consequences of interrupting the interaction of 14-3-3 with PLD. In addition, the difopein (dimeric 14-3-3 peptide inhibitor) plasmid construct, which can also be heterologously expressed and which consists of two WLDLE 14-3-3 recognition-type R18 peptides joined by a linker region (which can block both interaction sites within the 14-3-3 amphipathic groove) would yield valuable information about the functional interactions of 14-3-3 in GPCR signalling (Masters and Fu, 2001). Finally, the extension and application of these experimental techniques to other members of the GPCR superfamily would indicate whether 14-3-3 proteins provide a general functional role in the signalling mechanisms of G protein-coupled receptors.

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